Community acquired respiratory syncytial virus infections : detection by multiplex PCR and strain characterisation by partial G gene sequencing

Thesis

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Community acquired respiratory syncytial virus infections: detection by multiplex PCR and strain characterisation by partial G gene sequencing

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DECLARATION

I have not submitted any portion of the work referred to in this thesis in support of an application for another degree or qualification of this or any other university or institute of learning.

The work carried out in this thesis was carried out by myself unless otherwise indicated in the text.

Part of this work has already been published:

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ABSTRACT

The aim of this project was to design an assay for the detection of respiratory syncytial virus (RSV) RNA extracted directly from clinical specimens. The assay was intended to address the question of whether RSV is a significant cause of respiratory illness in all age groups of the general community.

The amplification assay for the detection of RSV subtypes A and B was designed using primers located in the nucleocapsid gene. This RSV PCR was incorporated into a multiplex PCR together with primers specific to influenza A H1N1, H3N2 and influenza B. The multiplex assay was optimised and validated, and different amplicon detection methods were investigated with a view to develop a high throughput protocol.

The multiplex PCR assay was then applied to combined nose and throat swabs collected from members of the general community with influenza or influenza like illness, over a three year period (1995-1998). Analysis of these results revealed the co-circulation of RSV and influenza during the winters. RSV was shown to be an important contributor to respiratory illness in all age groups, being detectable in about 20% of patients with influenza like illness.

The RSV positive samples from the three winter seasons studied were processed to obtain sequence data suitable for molecular epidemiological analysis. A strategy to amplify and sequence the first variable region of the glycoprotein gene was developed. PCR amplification was successfully performed directly using stored clinical samples. Phylogenetic analyses of the amplicons revealed that different strain types circulated during each winter season.

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ABBREVIATIONS

°C	Degrees centigrade
µl	Microlitre
CDC	Communicable Diseases Centre
CDSC	Communicable Disease Surveillance Centre
CPHL	Central Public Health Laboratory
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate (any of the four dATP, dCTP, dGTP, dTTP)
EIA	Enzyme immunoassay
ELISA	Enzyme-linked immunosorbent assay
ELOSA	Enzyme linked oligo-sorbent assay
HA	Haemaglutinnin
NA	Neuraminidase
hr	Hour
Ka	Non-synonymous
Ks	Synonymous
l	Litre
LC	Lightcycler
LRTI	Lower Respiratory Tract Illness
ILI	Influenza-like-illness
min	Minute
ml	Millilitre
mM	Millimolar/millimole
PCR	Polymerase Chain Reaction
URTI	Upper Respiratory Tract Illness
RCGP	Royal College of General Practitioners
RNA	Ribonucleic Acid
rpm	Revolutions per minute
RSV	Respiratory Syncytial Virus
RT	Reverse transcriptase
RT-PCR	Reverse transcriptase PCR
sec	Second
Ta	Annealing temperature
Taq	*Thermus aquaticus*
temp	Temperature
Tm	Melting temperature
Tris-HCl	Tris (hydroxymethyl)aminomethane hydrochloride
Ts	Transition
Tv	Transversion

Chapter 1

General Introduction

General introduction

Respiratory illness is the most common reason for consultations with general practitioners in all age groups, and seasonal respiratory infection is an important cause of hospitalisation and excess mortality in the winter months (167). Infectious respiratory disease can be caused by several pathogens and the clinical presentations include influenza like illness (ILI), otitis media, sinusitis, laryngitis and bronchiolitis (215). Relating these symptoms to a specific pathogen may be difficult, which in turn makes diagnosis difficult (81). Prominent amongst the viral causes of respiratory illness are influenza (A and B), and respiratory syncytial virus (RSV).

Respiratory syncytial virus (RSV)

RSV is an enveloped, negative single stranded, RNA virus in the family paramyxoviridae, with a genome approximately 15,200 bp in length. There are two subgroups of RSV designated A and B, based on their reactions with monoclonal antibodies (173). The virus genome encodes 11 proteins (47) (14), with the F and G protein being the most antigenically important (fig 1.1). RSV is classified in the Order Mononegavirales, a group of viruses which have several properties in common. These include the single stranded negative sense RNA genome which is tightly associated with viral protein, and a replication cycle that takes place in the cytoplasm. There are four families in this order: Rhabdoviridae whose members include rabies virus and vesicular stomatitis virus (VSV); Filoviridae which include Ebola and Marburg; Bornaviridae and the Paramyxoviridae. The family Paramyxoviridae comprises two subfamilies: Paramyxovirinae and Pneumovirinae. There are three genera in the Paramyxovirinae family: Paramyxovirus, Morbilivirus, Rubulavirus, and there are two genera in the Pneumovirinae family, Pneumovirus and Metapneumovirus (175,196) RSV belongs to the genus Pneumovirus (fig 1.2), other members of this genus include bovine RSV (BRSV), ovine RSV (ORSV), caprine RSV (CRSV), and pneumonia virus of mice (PVM).

Figure 1.1 Diagrammatic representation of RSV virion

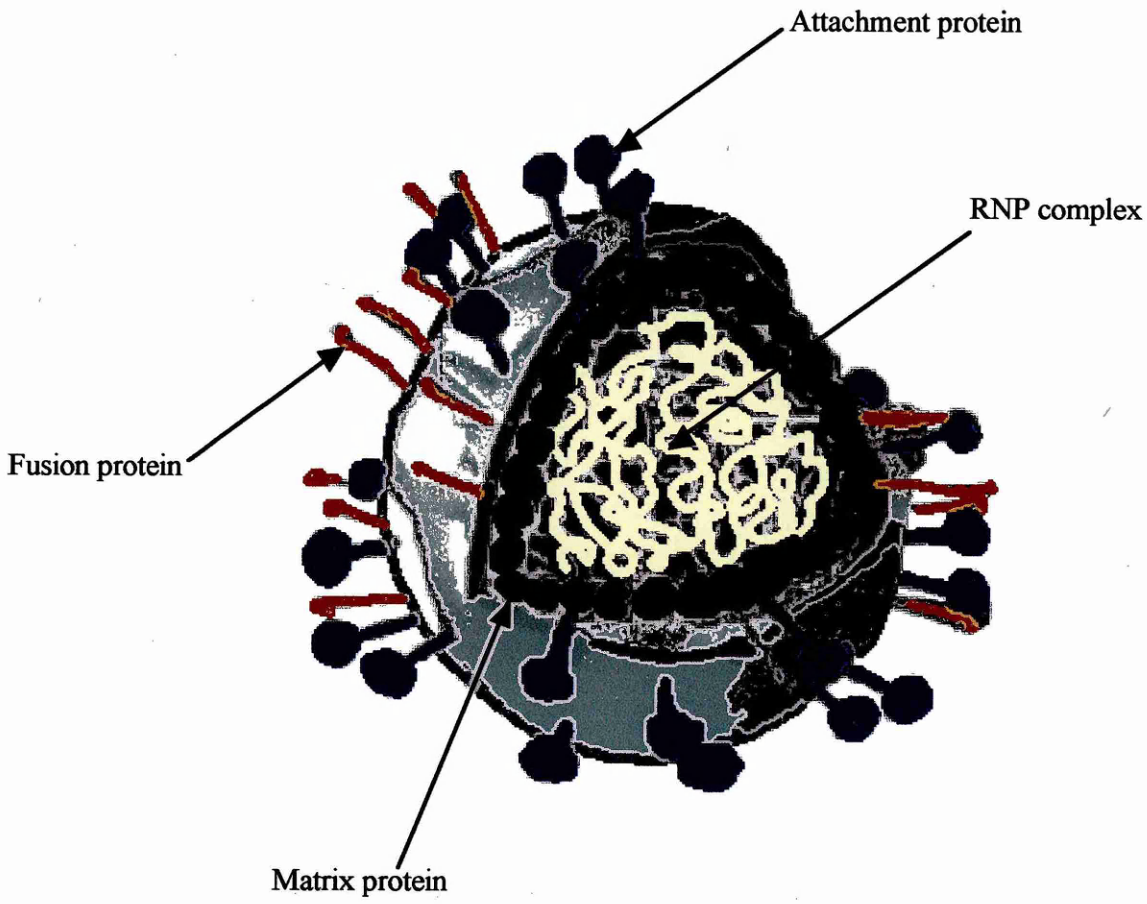
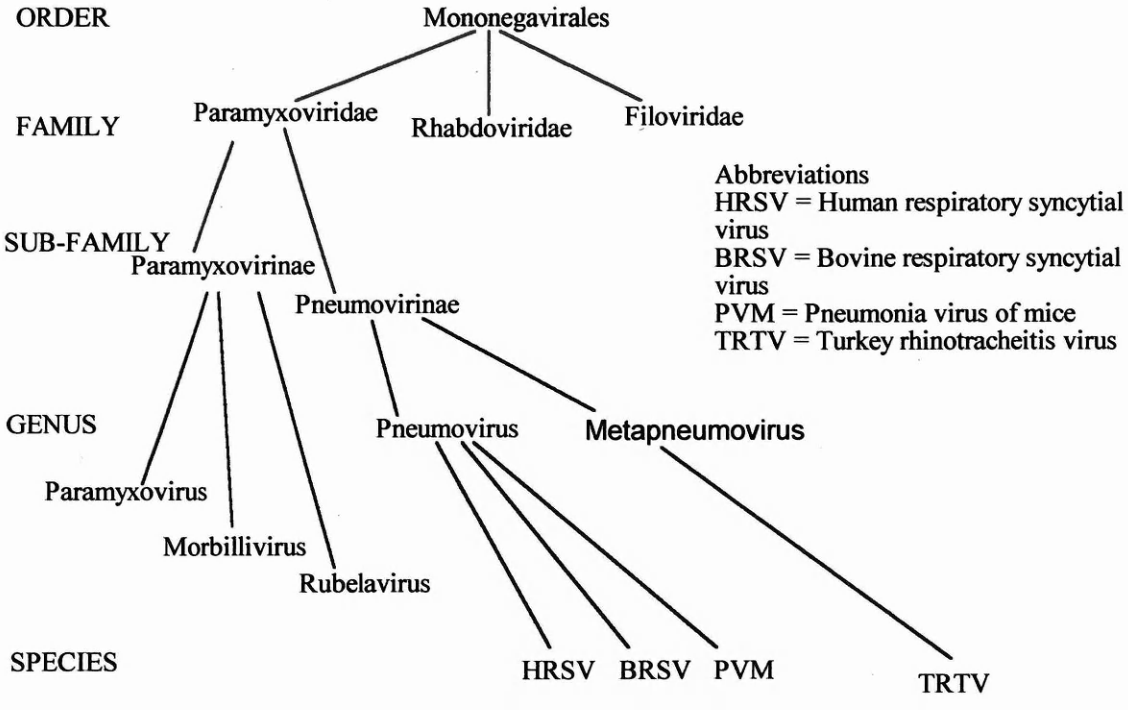


Diagram adapted from Warwick University internet site (www.bio.warwick.ac.uk/easton/)

Figure 1.2 Taxonomy of RSV



History

RSV was first isolated in 1956 from a laboratory chimpanzee displaying signs of respiratory infection (171). Shortly after this identical viruses were isolated from children in Baltimore (USA) and the characteristic syncytia formation that earnt the virus its name were observed (39). The virus was soon recognised as a severe cause of illness in infants and children all around the world (2, 30). Research into RSV is hampered by its instability, poor growth in tissue culture, and lack of an ideal experimental animal model (20,48).

Clinical features

RSV is recognised as an important respiratory pathogen in young children, and is estimated to be responsible for approximately 90,000 hospitalisations and 4,500 deaths in this age group annually, in the United States (50). RSV has also been demonstrated to be an important contributor to disease in the elderly (74, 180). The exact contribution of RSV to

the burden of respiratory disease in older children and the adult population is, however, unknown. Infection with RSV is known not to confer life long immunity, and re-infection occurs throughout life (172). RSV often presents with a variety of upper and lower respiratory tract symptoms but the best predictor of infection has been reported to be wheezing (176).

Replication cycle

The receptor to which RSV binds on the host cell is unknown (192). However as the virus does not possess a neuraminidase, it is probable that it is not sialic acid. RSV is believed to penetrate the host cell by fusing with the plasma membrane. The viral envelope then becomes incorporated in the host cell surface. The nucleocapsid of the virus is released in the cytoplasm during replication which, unlike influenza virus, does not require the nucleus of the host cell (48).

The exact mechanism of initiation and termination of RNA replication of the genome of the mononegavirales is not understood. Each gene has a conserved start and stop sequence that are separated by intergenic junctions of variable sizes (fig 1.3). These intergenic junctions are not present in the mRNA (48). The proposed mechanism for viral replication of non-segmented negative strand viruses is that the polymerase molecule binds the 3' end of RNA. The polymerase transcribes the RNA until it encounters a gene end signal, then falls off and binds again at the next gene downstream. Rearrangement experiments have shown that the position of a gene relative to the 3' end of the vRNA affects expression of it. The N gene of VSV is required stoichiometrically for genome replication. A move of one position downstream of VSV N gene resulted in a 15 fold decrease in viral yield (compared to wild type). A move of three gene positions downstream resulted in a 20,000-fold decrease in virus replication (254). This replication mechanism would explain why the proteins encoded by the vRNA nearest the 3' end are the most abundant.

It is speculated that as the polymerase molecules progress further down the RNA fewer of them re-attach and therefore, less gene product is seen for those proteins encoded by RNA nearer the 5' end. Recently Hardy *et al* (1999) have shown that the efficiency with which the polymerase terminates transcription of a gene is affected by the gene junction it encounters, with some gene junctions more efficient at this than others (102). It has also been observed that RSV exhibits "readthrough transcription" (103). In this mechanism the RNA polymerase does not fall off the RNA at a gene end signal, but carries onto the next gene. This mechanism enhances the production of genes further downstream, and it has been suggested that the M2 protein (ORF1) is involved (103). The M2 protein appears to regulate its own production through negative feedback, and although it reduces polymerase termination at every gene junction, the extent of antitermination activity differs at each (102). The junction with the SH and G gene (fig 1.3) showed the most efficient antitermination of polymerase (readthrough) and produced low levels of readthrough transcripts even in the absence of the M2 protein (102). The amount of transcriptional readthrough in RSV is high compared to other Mononegavirales, but these other viruses only contain five to six transcriptional units and, four to five gene end or stop signals. RSV has 10 transcriptional units and, nine stop signals, which may explain why it has evolved a mechanism to enhance transcriptional readthrough.

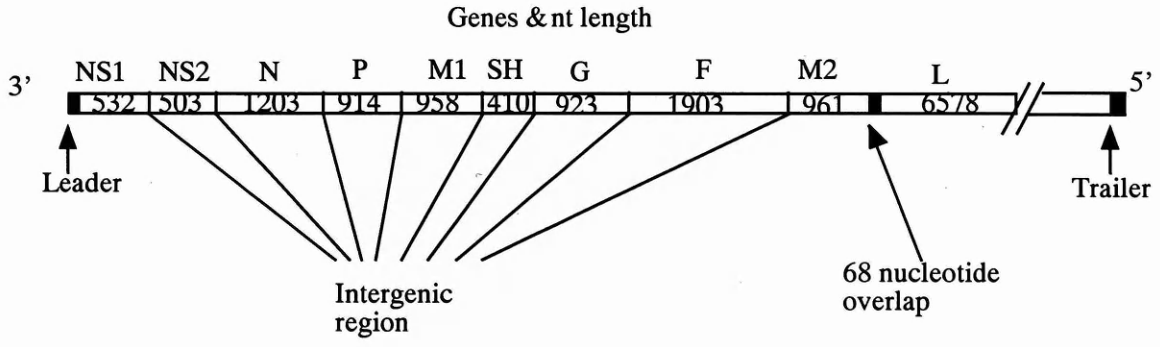
The intergenic junctions between genes NS1 and M2 are of differing lengths (fig 1.3). These different sequences appear conserved in length in the different strains of RSV, (142) but seem to play no role in regulating gene expression. A study where the intergenic regions were swapped, altered, or even deleted showed there to be no change in gene expression (142). Each gene of RSV has conserved gene start (GS) and gene end (GE) signals. The GS signal for the first nine genes is highly conserved and crucial to gene expression. The GS and GE signals are also transposable, in that when they are attached to a foreign sequence they will direct transcription (143). Saturation mutagenesis of each nucleotide of the GS signal in turn showed that all positions were important with the possible exception of position five (141). The same study also demonstrated quasi-templated initiation, where a mutation of position one of the GS signal only resulted in two thirds of the resulting mRNAs containing the mutant nucleotide. The rest of the mRNAs

species had reverted to the parent type with the correct nucleotide at position one (141). The GE signal is not as conserved as the GS signal, but it is critical for gene expression. The presence of the GE signal alone allows some gene expression, and removal of both GS and GE signals completely silence gene expression. This would suggest that entry of a polymerase at an internal site in the gene is unlikely (143).

RSV Gene structure

There are 10 genes in the RSV genome (fig 1.3), which in turn are transcribed into 10 mRNA species (47).

Figure 1.3 RSV gene order



NS1 to NS2	19 nucleotides	M to SH	9 nucleotides
NS2 to N	26 nucleotides	SH to G	44 nucleotides
N to P	1 nucleotide	G to F	52 nucleotides
P to M	9 nucleotides	F to M2	46 nucleotides

RSV genes

NS1 and NS2

The NS1 and NS2 genes encode non-structural proteins whose precise function is unknown. Both the NS1 and NS2 proteins are unique to pneumoviruses. The NS1 gene is 503 nucleotides long with 375 nucleotides constituting an open reading frame (ORF). There is 92% sequence identity between RSV A and B strains in the NS1 gene (126). The NS1 protein is 139 amino acids long, and is abundant probably due to its location in the genome, near the 3' end (fig 1.3). Recent studies have shown that the NS1 protein appears to have an inhibitory effect on both RNA transcription and replication, which is not limited

to RSV (7). Expression work with a parainfluenza 3 (PIV3) minigenome analog showed that PIV 3 transcription and replication was inhibited by the RSV NS1 protein (7).

Proposed Function of the NS2 gene

The NS2 gene is 523 nucleotides long and contains an open reading frame of 420 nucleotides. There is 87% sequence identity between RSV A and B strains (126). Recently Teng and Collins (1999) created RSV mutants which either had termination codons scattered throughout the NS2 gene or completely lacked the NS2 gene (231). Using a reverse genetics system to express the altered viruses they were able to show that lack of the NS2 gene did not prevent the virus from replicating in cell culture; rather the growth of those viruses which lacked the NS2 gene was attenuated. This study provided some evidence that the NS2 protein may positively regulate the transcription and/or regulation of the other RSV genes, or be involved in the transport of the virus proteins to the cellular membrane (231).

N gene

The N gene encodes for the nucleocapsid protein and is 1203 nucleotides long with an open reading frame of 1176 nucleotides. The N gene is involved in RNA replication, together with the P and L genes and another unidentified factor (97). It is highly conserved between and within RSV subtypes and strains, with a sequence identity of 96% (126). The N gene has therefore been the target for study on the classification and subtyping of strains of RSV (28, 223).

P gene

The P gene encodes a major phosphoprotein, and has a length of 914 nucleotides. The P protein is thought to have two major functions, one as a transcription factor and the other as a replication factor. It is phosphorylated mainly on serine residues (10) and sequence information shows two conserved regions suggesting a modular organisation of the P protein (48).

M 1 gene

The M gene is 958 nucleotides long and encodes a matrix protein. In non-segmented negative strand viruses M genes are believed to have two functions: to trancriptionally inactivate the nucleocapsid, and to assist the interaction of the nucleocapsid and the envelope. As RSV is a member of this order (fig 1.2) it is assumed the M gene in RSV also has these functions (48). Recently it has been suggested that the M protein may be involved in virus motility by some unknown interaction with cellular actin (236).

SH gene

The SH gene is 410 nucleotides long and encodes a small hydrophobic glycoslylated membrane protein whose function is unknown. In experiments where the SH gene has been deleted from the genome the virus still grows in cell culture (18). These experiments would seem to indicate that the SH protein plays no or little role in viral replication *in vitro*.

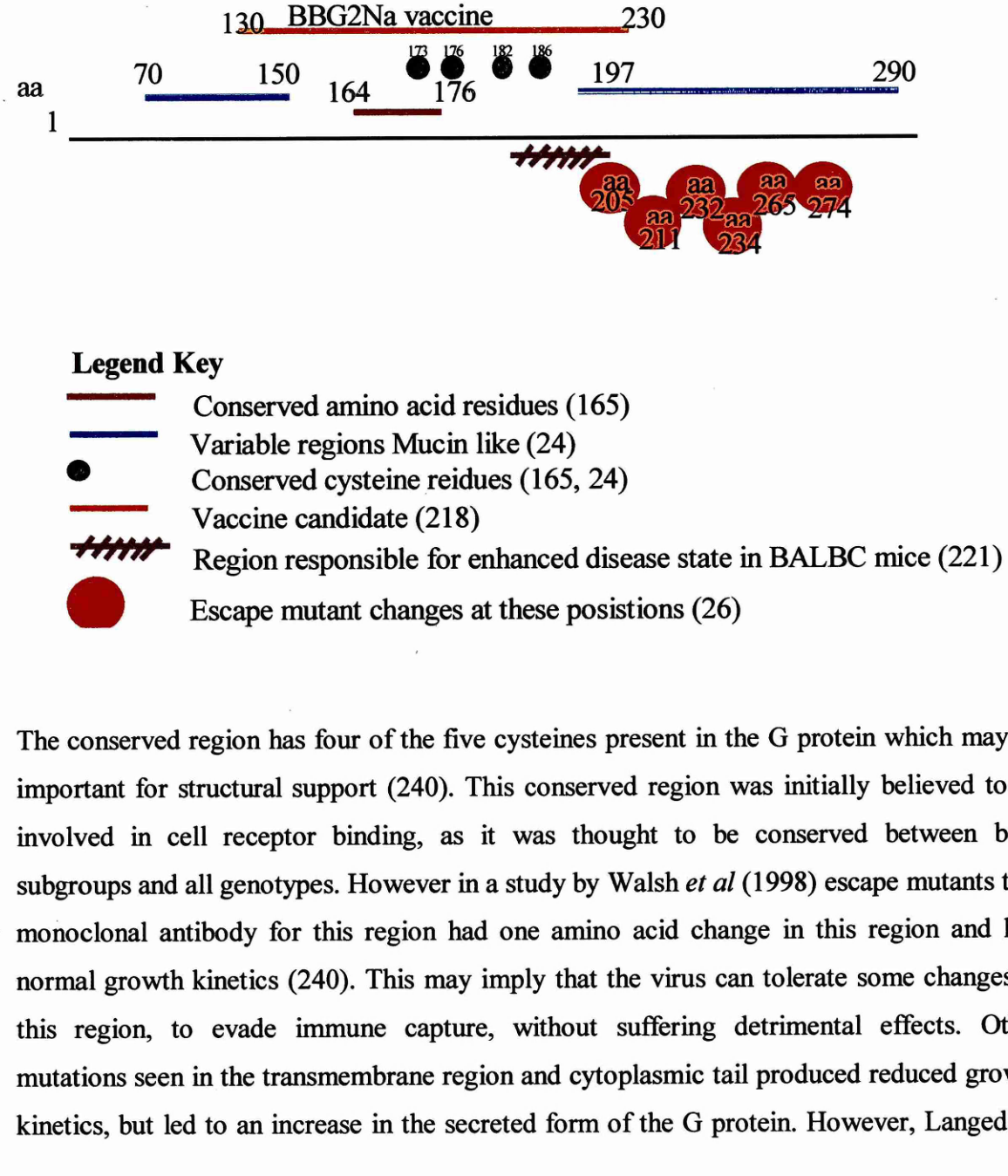
RSV G gene

Structure and function of the G gene

The G gene is 923 nucleotides long and codes for a processed transmembrane protein of 33 kDa. After post-translational modification the mature protein is around 90 kda (239) and has an unusually high content of serine, threonine and proline. It is also highly glycosylated with both N- and O-linked oligosaccharides, which are more commonly associated with mucus glycoproteins (253). The G protein of RSV is believed to be involved in attachment to the cell membrane (151), and it is one of two main antigenic determinants of the virus. There are three main regions to the G protein (fig 1.4): the intracellular, trans-membrane and extracellular regions. The extracellular region can also be further split into two variable regions separated by a highly conserved region (24). The cellular receptor for the RSV G protein has yet to be identified (226). The G protein is a type II glycoprotein, with a N-terminal signal transmembrane region (24). It is highly glycoslyated, which makes the protein similar to mucin like proteins which are excreted by epithelial cells. The nucleotide sequence and subsequent amino acid length of the G protein varies between the different subtypes and strains of RSV. The amino acid length can vary between 292 and 299 amino acids, within RSV subgroups (23). The G protein has a predicted molecular mass of around

33 kDa (239). Post translational modification includes the addition of several N-linked and O-linked carbohydrate side chains which results in a mature protein of around 90 kDa (239). Recently it has been suggested that the extracellular domain of the G protein has a non-glycoslyated region which contains a cysteine noose (95).

Figure 1.4 Diagrammatic representation of the G gene of RSV



The conserved region has four of the five cysteines present in the G protein which may be important for structural support (240). This conserved region was initially believed to be involved in cell receptor binding, as it was thought to be conserved between both subgroups and all genotypes. However in a study by Walsh *et al* (1998) escape mutants to a monoclonal antibody for this region had one amino acid change in this region and had normal growth kinetics (240). This may imply that the virus can tolerate some changes in this region, to evade immune capture, without suffering detrimental effects. Other mutations seen in the transmembrane region and cytoplasmic tail produced reduced growth kinetics, but led to an increase in the secreted form of the G protein. However, Langedijik

et al (1996) concluded from their studies that peptides corresponding to this reported conserved region could be used successfully in a diagnostic assay (145).

Sequence divergence in the G gene

High divergence in the sequence of RSV G is seen between the subtypes of RSV. Divergence of up to 47% has been reported between the two subgroups, with 57% divergence reported in the extracellular portion of the G protein (127). Intrasubgroup variation has been reported to be as much as 20% (26, 225). Over half of the nucleotide changes in the G gene are non synonymous, suggesting that there is a selective pressure for change in this region (225). The cytoplasmic tail and transmembrane portion of the G protein appears to be less divergent, having 84% identity (127).

Membrane associated and secretory forms of G

The G mRNA has one ORFs coding for the entire G protein, a truncated G protein is cleaved at amino acid 66 and secreted (109, 205). This protein lacks the entire transmembrane region of the intact G protein and is not membrane associated. The function of this secreted form of the G protein is unknown. It may be that the truncated version of the G protein binds any circulating antibodies thus preventing them from binding to the virus particle itself (239).

The secreted G protein is highly antigenic, with the membrane anchored form of the protein producing a different immune response from the secreted form (129). It has been suggested that the secreted form of G is a strategy for evading and modulating immune responses, and is an important factor in disease severity (129). The immunity produced by the G protein is subgroup specific (27). RSV mutants which lack the SH and G surface protein can still replicate *in vitro*, which suggests that the G protein may not be the major surface protein involved in virus entry (132). Transcriptional readthrough is present at the SH/G gene in the absence of the M2 protein (102). This may have evolved to enhance the production of the RSV G protein. This would support the hypothesis that the secreted form of the G protein is produced to 'mop up' any antibody present, giving the virion an increase chance of penetrating and infecting the host cell.

F gene

The F gene is 1,903 nucleotides long and codes for a transmembrane surface glycoprotein. It is responsible for fusion to the host cell membrane and mediates virus penetration. It is also responsible for the syncytia formation observed in cell cultures infected with RSV (156, 240). The F protein gene is highly conserved and highly antigenic. Immunity produced via the F protein confers protection against both subtypes in animal experiments (27). It possesses two subunits F1 and F2 joined by a disulphide link; the F1 subunit is more highly conserved than the F2 subunit (156). It is believed that the F protein is the major protein involved in virus entry (132). Monoclonal antibodies to the F protein are currently being investigated with the aim of developing better therapies for RSV infection (73, 211).

M2 and L genes

The M2 gene is 961 nucleotides long and is unique to pnuemoviruses (48). It is suggested that this protein is important in virus transcription, and is believed to be involved in the balance between RNA replication and transcription (14). The L gene product is a nucleocapsid associated protein. The gene is 6,578 nucleotides long with the start codon for it within the M2 gene. These two genes overlap by 68 nucleotides (49).

Evolution of RSV

The amount of variation which is seen in RSV G may be analogous to the extent of change seen in the HA of influenza (26). This phenomenon of antigenic drift in influenza gives the virus an ability to re-infect individuals. Much like influenza, analysis of RSV strains has shown that they fluctuate over a number of years. A study by Cane *et al* (1994) showed yearly variation in the predominant circulating RSV strain (22). This would support the hypothesis that variation in the G gene confers an advantage to the virus allowing re-infection to occur. In contrast, a study by Johansen *et al* (1997) found that strains, isolated from hospitalised children, circulating in epidemics in Denmark were related to those isolated over twenty years previously (125). This lead them to the conclusion that the predominating strain is selected from a global pool of genetically stable strains, and not due to progressive evolution of the virus (125). However, other studies on RSV isolates from

different parts of the world, all from hospitalised children, support the hypothesis made by Cane *et al* (1994) that similar strains of viruses co-circulate at the same time globally, and represent progressive evolutionary changes in the virus (22, 25, 88, 157). Most reports of RSV sequences are on isolates from temperate countries, however one study of RSV isolates from a tropical country (Gambia) has recently been reported (30). All isolates studied were obtained from infants. Four epidemics of RSV were analysed by sequencing of the G gene, and epidemics were found to be seasonal and associated with the rainy season. Variation in the predominant subtype between the seasons was demonstrated, with some strains similar to those circulating elsewhere in the world (30). However, some isolates were unlike those identified elsewhere, and likewise a commonly observed strain identified in the UK was not identified in these Gambian isolates (30).

Detection Methods for RSV

The clinical presentation of RSV can be similar to other causes of respiratory illness, and diagnosis of illness based entirely on clinical presentation shows a lack of conformity in results (215). This makes diagnosis of RSV difficult when based only on this criterion. There are several laboratory tests for RSV, many of which can be purchased commercially (Table 1.1). Cell culture can be used to detect RSV: typically Hep-2 cell lines are incubated at 37°C with virus adsorption at 34-35°C. However the success of this test depends on the sample type used, and growth of RSV from clinical samples can be difficult. RSV is a labile virus and as such requires cold storage to retain infectivity and samples stored at -20°C which are subjected to freeze/thaw cycles will lose infectivity. Studies comparing methods for detection of RSV have shown that methods other than cell culture often prove to be more sensitive; these include shell vial and ELISA tests (189, 198). More recently molecular methods for detection of different virus genomes have been described which can provide a more sensitive assay and sequence information about the viral genome (121, 223, 227).

Table 1.1 Diagnostic methods for RSV detection

Method	Sample required	Estimate Cost (per sample)	Time Taken	Advantages	Disadvantages
Culture	Nasopharyngeal aspirate, Nose and throat Swab, Bronchoalveolar lavage	£10-20	7 Days	Whole virus measured Virus recoverable "Gold Standard"	Requires infectious virus Highly skilled
IF	Nasopharyngeal aspirate, Nose and throat Swab, Bronchoalveolar lavage	£5	2 Hours - 1 Day	Rapid	Requires intact cells Highly skilled No virus recoverable Specialised equipment needed Labour intensive Non-specific Numbers limited
Antigen	Nasopharyngeal aspirate, Nose and throat Swab, Bronchoalveolar lavage	£5-10	15 mins - 1 Day	No specialised facility needed Can be "near patient" testing Low skill Rapid	No virus recoverable Poor sensitivity/specificity? Often non-proven technology
PCR	Nasopharyngeal aspirate, Nose and throat Swab, Bronchoalveolar lavage, unfixed post-mortem tissue	£20-30	1-2 days	Sensitive Allows further molecular analysis	No virus recoverable Specialised equipment / laboratory needed Highly skilled
Serology	Serum	£5	2# Days	Sensitive and specific	Retrospective, paired samples needed
CF*	Serum	£5	2 Days		Insensitive, retrospective, paired samples needed

* Complement Fixation

Acute and convalescent sera required (14 days)

Animal models for RSV

A wide range of clinical symptoms are associated with RSV infection, with severe disease in infants (115). Although severity of illness is not known in the adult population it is unlikely that this disease on its own would produce a life threatening state. As the symptoms and severity of disease vary it is unlikely any one animal model would provide an accurate reflection of disease for every population affected by RSV. There are several animal models available for studying RSV infections. For example the chimpanzee, from which the virus was first identified, has been used (133). The validity of results from experiments with chimpanzees may be questionable as no evidence of any pulmonary infection has been documented. Also, there is no evidence that formalin inactivated vaccine produces an enhanced disease state, as seen in humans (discussed later in this chapter) (20). Other animal models include the cotton rat, which is susceptible to RSV infection throughout adulthood, although lack of suitable strains and availability are a disadvantage (20, 224). Mice would be advantageous due to their availability and ease of handling, however they are not particularly susceptible to RSV infection. Recent developments include the treatment of mice with an immunosuppressive agent prior to infection to increase susceptibility (224). Calfs, ferrets, guinea pigs and hamsters are used or have been considered, each system having advantages and disadvantages. The three main animals used currently despite their limitations are the mouse, cotton rat and primate (20 , 76, 128).

RSV Subtypes

The two subgroups of RSV can be further separated into different strains or lineages. Subgroup A has been genotyped into 6 different lineages based on restriction fragment length polymorphism (RFLP) analysis of the N gene and sequencing of the SH gene (22). Subtype A has also been further broken down into 22 'clades' (191). Subgroup B has been divided into two different lineages on the basis of RFLP on the NP genes (27) and into 6 lineages by analysis of the G gene (discussed in chapter 5) (191).

Re-infection with RSV has been shown to occur in a study by Mufson *et al* (1987) in which repeat infections with RSV in children were studied (172). Of ten children who had a RSV A infection one year, four were re-infected with RSV A in the second year, whereas the rest

experienced RSV B infections. This would suggest that an infection with one RSV subtype will not prevent subsequent infection with the other RSV subtype, or even with the same subtype again. In one study the majority of children tested had experienced two RSV infections by the time they reached one year old (94). It has also been demonstrated that previous infection with RSV does not guarantee that symptoms of RSV disease upon re-infection will be less severe, and the repeat infection has been shown to last almost as long as the primary infection (234).

Prevalence and severity of disease associated with a particular subtype

Answering the questions: 'Which subtype is the most prevalent, and which causes the most severe disease?' can be very complicated. Studies designed to answer these questions have often arrived at conflicting results. These studies have usually been conducted on a specific subset of the population, particularly hospitalised infants. Studies conducted over a period of many years have found that RSV A is the most predominant overall, however the individual winter seasons vary as to the predominant subtype (100, 110).

A recent study which looked at the severity of RSV illness in hospitalised infants used various markers to measure severity of disease in infants (115). These markers were both clinically and immunologically determined. It was concluded that in this population RSV B caused more severe disease than RSV A. In contrast, however, McConnochie *et al* (1990), who studied severity of disease in infants using clinical markers as indicators of severity, found RSV A to cause more severe disease (166). Another study conducted on hospitalised infants also concluded that RSV A caused more severe disease than RSV B (241). As most of these studies were conducted on a specific subset of the population, usually hospitalised infants, specific information on RSV severity in other subsets of the population, and in the general community is lacking.

Pathogenesis of RSV

The incubation period for disease after receiving an inoculum of RSV infectious particles has been estimated to be five days (64). The virus replicates to high titre in the nasopharynx, and although the mechanism of viral spread is unclear it is presumed that the

virus travels directly through the cells of the respiratory epithelium (64). RSV can spread from cell to cell, without having to enter from outside the host cells, by inducing syncytia formation. There is some evidence from animal models to suggest that the virus may sometimes enter into extracellular spaces (64). Further evidence of the presence of RSV RNA (both genomic and messenger) outside of the respiratory tract, has recently been reported (183). Viral RNA was identified in the arterial blood of children, but it is unclear if the virus is capable of replication in peripheral blood cells as replicating virus has not been recovered (183). Another possibility is that the virus infects macrophages and 'hitchhikes' down into the lower respiratory tract (64). Ulloa *et al* (1998) recently described another possible motility mechanism employed by RSV, through the use of cellular actin (236). Actin can be found in purified RSV virions and interactions between cellular actin and virus components may promote the formation of cytoplasmic extensions from the host cell containing virus particles *in vitro* (236).

As with many infections, disease progression is thought to be a combination of the toxic effects of the virus alongside the body's own immune response to the infection. RSV primarily infects the respiratory epithelium, which leads to increased production of mucus. Infected ciliated cells are sloughed off into the small airways and, in combination with the mucus, form plugs. This may account for the increased fatality in young infants as the airways are smaller and more easily blocked (64). The increase in mucus and loss of ciliated cells increases the chances of secondary bacterial infection.

First vaccine trials

A formalin inactivated whole RSV vaccine was developed and three trials performed in the United States in the late 1960s (85, 130, 137). All three of the trials involved inoculating the participants with the vaccine and then infecting controls and vaccine recipients with RSV. The outcome of these trials was disastrous with the resulting disease in the vaccine recipients being far more severe than the controls (137). The trials ended with two infants dying in the vaccinated group, whereas none died in the control group.

The immunological mechanisms for this vaccine enhancement of disease have now been elucidated, and it is believed that the vaccine only caused a partial T cell response that resulted in exaggerated pulmonary inflammation after challenge with RSV (60). Live virus induces a TH-1 type response whereas the killed vaccine induced a TH-2 type response. The vaccinees produced a strong CD4+, response but a weak CD8+, response which resulted in exaggerated pulmonary inflammation upon challenge with the wild type RSV, with the CD4+ cells dominating the lung (60). It is possible that the inactivated vaccine altered the secretory and neutralising antibody response and upon reinfection the virus could replicate freely and spread to the lung. This may have been due to the route of inoculation of the inactivated vaccine, which did not promote an IgA response. The CD4+ memory cells would have been amplified, and the shifted pattern of released cytokines could have enhanced inflammation producing more severe disease (99). It has also been demonstrated that eosinophils are recruited into the respiratory tract in response to RSV infection. They have been shown to possess antiviral activity; however when over recruited they may lead to exaggerated forms of disease reviewed by Domachowske and Rosenberg, 1999 (64).

Immunity to RSV

The role of immunity to RSV infection is still not clearly defined. It would appear that there is a fine balance between the involvement of different immune cell types. Both serum and secretory antibodies are produced in response to RSV infection (64). Maternal immunity passed to the infant may be very short lived, lasting only one to two months. Therefore infants are susceptible to infection from as young as one month after birth, unlike other forms of maternal immunity which will protect the infant for considerably longer (40). Breast fed infants may have the added benefit of maternal antibody in the form of colostrum (64). In infants, antibody titres produced to RSV infections are low, possibly due to the immuologic immaturity of the infant or the suppressive effects of maternal antibody. Although the humoral immune system clearly plays a role in controlling RSV infection, it is believed that it is the cellular arm of the immune response that is responsible for clearing the virus once infection is established reviewed by Domachowske and Rosenberg, 1999 (64). The most convincing evidence that cellular immunity is intimately involved comes

from the early trials of a formalin inactivated vaccine (60). It is interesting to note that RSV G fails to promote a CTL response, normally a TH-1 response with IF-gamma and IL-2 are seen with a viral infection (64).

Immune response to the G protein

The immune response generated to a RSV infection is complex and not fully understood. However, studies to elucidate the mechanisms behind vaccine enhanced illness provided some insight into the immune response to a RSV infection. The huge amount of variation seen in the nucleotide sequence of the G gene suggests that there is an immune or selective pressure on this region (26). Any vaccine designed for use against RSV infection needs to elicit the right kind of immune response, to not only protect against re-infection but also to guard against enhanced illness upon subsequent challenge.

Types of immune response to RSV infection

The host response to many infections involves a combination of humoral and cellular immunity, and RSV generates both of these responses in a natural infection. The generation of different types of immune response is seen with the different proteins of RSV, with the F protein activating a CD4+ Th-1 (interferon gamma and Il-2) type response (222), whilst the M2 protein generates a CD8+ response in the mouse animal model (221). The G protein of RSV generates a CD4+ Th-2 type response (Il-4 and Il-5) response with eosinophilic influx into the alveolar space (221, 222). This leads to a state of enhanced illness with increased severity of disease. Recently the region of the G gene believed to be responsible for the enhanced disease state with eosinophilic influx, was mapped to a 10 amino acid stretch (amino acids193-203) in the second variable region of the G gene (221). This region was shown to be the cause lung eosinophilia and weight loss in the murine animal model, but was not necessary for the generation of a protective immune response.

In contrast, a study by Tebby *et al* (1998) identified a different amino acid region (aa184-198) which was associated with eosinophilic influx in BLAB/c mice (229). In this study the deletion of CD4+ cells prior to the administration of the peptide from amino acid 184 to amino acid 198 eliminated the eosinophilic influx, whereas the deletion of CD8+ cells prior

to administration of the peptide had negligible effect. Of the six blood donors tested for antibodies to this peptide, three showed a response. This demonstrates an association between the 184-198 peptide and the CD4+ Th-2 type enhanced response (229).

RSV primarily infects the epithelial cells of the lower and upper respiratory tract. A recent study by Hussell *et al* (1997) showed the critical role that the CD8+ cells play in regulating the CD4+ response to RSV infection in BALB/c mice (117). This particular strain of mice only produces a CD4+ Th-2 type response to RSV G protein which results in the lung eosinophilia pathology. Administration of CD8+ cells reduced this enhanced disease state, which provides evidence for their role in regulating the Th-2 driven enhancement of disease (117). The same study indicated that depletion of interferon gamma (IFN-γ) also resulted in the enhanced disease state, suggesting that IFN-γ plays a role in the regulation of the Th-2 pathology. In support of this, a study by Srikiatkhachorn *et al* (1997) demonstrated the possible role that CD8+ cells have in the regulation of CD4+ cells and esoinophilic influx during RSV infection (222)

Interleukin-8 (IL-8) is a powerful chemotatic cytokine, which is present in elevated levels during RSV infection (139). A study which measured the levels of IL-8 in response to priming with either intact RSV or purified RSV G protein showed that when RSV G protein was present in high concentrations, the release of IL-8 was inhibited (139). This may be a function of the secreted form of the G protein, in inhibiting IL-8 release and therefore the recruitment of other cytokines, which will in turn limit the immune response. Further evidence for the role of cytokines in regulating RSV infection is accumulating, with IL-6, IL-10 and RANTES (regulated on activation, normal T cell expressed and presumably secreted) detected in elevated levels during an RSV infection (216).

Recently, the antiviral activity of a human eosinophil-derived neurotoxin (EDN, RNase 2) has been demonstrated for RSV (63). EDN, RNase 2 is a protein secreted by eosinophilic leukocytes which mediates the destruction of extracellular RNA. The interaction of this molecule with RSV has recently been shown to be specific to RSV (63). This provides further information on the human immune response to a RSV infection.

Regions against which immune response is directed on the G protein

The carboxyterminal of the G protein (second variable region) does initiate an immune response, which offers an explanation for the extent of variability seen there, however it is not clear whether the antibodies to this region are protective (29). Studying the immune response to various regions of the G protein, especially the variable regions, requires the knowledge of the infecting genotype. The antibody response to the carboxyterminal region, and to linear epitopes from this region, can be specific to the infecting genotype (23). Cane *et al* (1997) reported that the linear epitopes in the carboxyterminal region of the G protein, recognised by primary infection sera, were concentrated around potential N-glycosylation sites (23). Alteration of the N glycosylation of this region may provide a mechanism by which the virus can escape immune detection, as glycosylation differences due to the types of cell used to grow the virus can allow the virus to escape recognition by murine monoclonal antibodies (23, 90). It was also reported that changing the linear epitopes by one amino acid could eliminate any recognition by the sera raised against the unaltered form (23).

Re-infection with RSV

Re-infection with RSV occurs throughout life and one explanation could be that the variation which is seen in the RSV G protein forms an escape mechanism for the virus to evade the host immune system. The large number of coding changes which are seen in the variable regions of the RSV G gene support the theory that there is immune pressure, and therefore selection on this region of the genome. However, in a study conducted by Sullender *et al* (1998), two strains of RSV A were isolated from sequential infections in children (226). Analysis of the G protein showed up to 15% variation in the amino acid sequence of the viruses isolated from the children. However, studies of the viruses in animals did not provide any support for the hypothesis that G protein variability contributed to their ability to re-infect. As the cellular attachment receptor for G has not yet been determined, and may differ between species, it is difficult to definitively rule this possibility out as an escape mechanism employed by the virus.

The ability of RSV to re-infect may be due to two reasons: either immunity acquired from contact with RSV is not protective; or re-infection occurs with a different type of the virus. As immunity gained by exposure to RSV is not fully understood, the possibility that it is the immune response which is fading and allowing re-infection complex. However, it is known that antibody responses to RSV infection are made, but the mechanisms by which these are unable to protect against subsequent re-infection are unclear.

The possibility that re-infection occurs with different subtypes or strains which are not recognised by the immune system is equally difficult to explore. This is simply because sets of samples collected throughout an individuals life time are impossible to obtain. Research conducted where re-infections have been studied have only focused on the two subtypes of RSV, and not any variation within the strains. In a study on re-infection with RSV it was noted that infection with one subtype would not confer immunity to the other subtype the following year (172) or to the same subtype (172, 226).

Prevention and treatment

Current vaccine development

There are some major considerations when developing a vaccine for RSV. The target population for vaccine studies has centred around infants, as they are the most likely to have a fatal RSV infection. Most of these infections are likely to occur when the infant is less than 12 months old (94). Due to this maternal immunisation may be of benefit, but there are certain drawbacks with this approach. Premature infants may not receive placental transfer of IgG, as this happens late in the third trimester of pregnancy. Additionally, maternal immunity may not stimulate a cell mediated immunity in the infant. There are also certain cultural and safety issues involved in vaccinating pregnant women. Other groups for which vaccination would prove beneficial are the hospitalised, and the elderly. Several incidents of RSV infections in these groups have been reported (74).

Live attenuated vaccines

These vaccine types are most likely to imitate a natural infection and therefore illicit the correct immune response. Nasal administration should produce both systemic and local immunity, perhaps providing the best defence against re-infection. Various methods have been employed to attenuate vaccine candidate strains, including repeated passage, chemical and site directed mutagenesis, and reverse genetics reviewed by Dudas and Karron 1998, (67). Viable virus has now been recovered from cDNA transcripts *in vitro* which may pave the way for a 'designer' vaccine to be produced (123). Mutations in the attenuated viruses at many genomic sites will reduce the possibility of reversion to wild type and as such should be incorporated.

Earlier tests of cold passaged (cp) or temperature sensitive (ts) mutant vaccine candidates showed little promise. The viruses produced were either over attenuated and would not replicate *in vivo* or were under attenuated, and reversion to wild type was seen with transmission from test subject to placebo (67). No vaccine enhanced disease was witnessed with these trials. Animal trials of ts and cp mutants have proven more encouraging (133) and further testing is needed of these vaccine candidates.

Some testing of chemically modified viruses showed underattenuation with unacceptable levels of disease caused in healthy adult volunteers (133). Other mutants created by this method have performed with greater success and currently one of these (cpts248/404) appears to be safe and immunogenic, and has a stable ts phenotype (133). Bovine RSV will protect rodents, owls and monkeys from infection with human RSV, and it has also been considered as a possible vaccine candidate. However bovine RSV does not protect chimpanzees from human RSV infection and it has now been abandoned as a vaccine candidate (56). A combination of ts attenuation and reverse genetics was used by Whitehead *et al* (1999) to further attenuate an RSV A vaccine candidate (256). This involved the addition of a mis-sense mutation in the L gene which increased the temperature sensitivity and attenuation of the virus.

Vaccines based on RSV G protein

As the RSV G protein plays a pivotal role in immunity to RSV infection, several approaches for a vaccine against RSV based on the G protein have been tested. As the G protein induces subtype, and maybe even strain specific responses depending on the epitope recognised, any vaccine based on this region needs to provide as much coverage as possible. A vaccine currently in Phase I clinical studies contains the conserved region of RSV G protein (amino acids 164-176) plus part of the first variable and second variable regions (fig 1.4). This is fused to the albumin binding region of *Streptococcal* protein G (BB), to enhance its half-life when expressed in *E. coli* as a non-glycoslylated protein. The adsorption of this to alum (A1OH) makes the vaccine candidate (BBG2Na) more immunogenic, and has shown encouraging results in mouse animal models (195). This study also provided some evidence that glycosylation of the G protein was not necessary to induce a protective response, in contrast to the findings of Cane which suggested that a mechanism for immune escape is alteration of glycosylation at different sites (23, 90).

Further testing of this vaccine candidate in neonatal mice showed that immunisation with BBG2Na protected against RSV infection despite the immunologic immaturity of the recipient. In the presence of maternal antibody the route of vaccination for BBG2Na did not seem to be of importance (218). As a potential target group for the administration of vaccine may be infants, the development of a vaccine which can illicit a protective immune response in the presence of maternal antibody and despite immunologic immaturity would be extremely desirable. Also, the administration of BBG2Na with the adjuvant induced a more balanced Th-1/Th-2 type response than previously seen (195, 218).

Another vaccine candidate was produced from a cDNA clone containing all genes of the A2 long strain of RSV plus the G gene of RSV B inserted at the A2 F/M2 intergenic region (rRSVA2(B-G)) (123). The addition of plasmids encoding the N, P and L genes were required to recover infectious virus, which showed stable expression of the chimeric DNA. Recently Collins *et al* (1999) reported that the M2-1 protein was also required as a separate plasmid for efficient recovery of cloned virus (45). The growth of rRSVA2(B-G) was slightly attenuated, which may be due either to the increased genome length or to the

alteration of the M2 and L genes which were placed further downstream of the inserted gene (123). The addition of the M2-1 protein may also aid in the recovery of the recombinant virus (45). The chimeric RSV clone rRSVA2 (B-G) expressing the G protein of both RSV A and B may be a potential vaccine candidate.

It is clear that the immune response to the virus is complex and the mechanisms by which the virus can cause reinfections are likely to also be complex. Due to the extent of immune driven variability seen in RSV G it is clear that this must convey some sort of advantage to the virus.

Subunit vaccines

Several types of subunit vaccine have been developed and tested including baculovirus-expressed proteins of RSV, RSV F adsorbed to alum, and immuno-affinity purified RSV F proteins. The subunit vaccines usually contain either RSV proteins F or G or a combination of the two (56, 247). Another type of subunit vaccine candidate involves the combination of a RSV protein portion with a foreign adjunct. Libon *et al* (1999) combined the serum albumin-binding region of streptococcal protein G with the second variable region of RSV A G protein (152). This fusion of the two proteins produced a strong specific antibody response to RSV A in mice.

Aerosol delivery

A subunit vaccine based on RSV F protein with adjuncts was recently tested intranasally in mice (230). This method of immunisation may be better tolerated and provide local and systemic immunity.

Vaccinia delivery systems

Recombinant vaccinia viruses expressing nine RSV proteins have been evaluated in mice, and the recombinant F and G proteins found to provide long lasting protection against wildtype virus (51). However, when tested in chimpanzees low immunogencitiy was seen. Adenovirus vectors have also being developed and tested, but have met with similar results (116).

cDNA-derived vaccines

More recently infectious virus was recovered from cDNA clones of RSV (46). This technology may enable the engineering of mutant attenuated viruses which are genetically stable. Also Collins *et al* (1995) have recovered infectious virus from cDNA clones which expresses a foreign gene (46). This may allow the development of a vaccine which contains the antigenic components of both RSV A and B, as has recently ben reported by Whitehead *et al* 1999, (257)where a chimeric virus was created containing the F and G proteins from both RSV subtypes. Also the inclusion of immunomodualting genes, such as the gene for IL-6, which may enhance immunogenicity of the vaccine in young infants may be helpful (17).

DNA vaccines

Another possibility for prevention of RSV illness is the use of DNA vaccines. A DNA vaccine is a plasmid which encodes an antigen which when inside the host is capable of expression (57). The production of these vaccines is cheap and relatively easy and so is advantageous over conventional vaccine formats. There are various safety concerns which need to be addressed with DNA vaccines including the possibility that the plasmid and foreign antigen DNA may integrate into human chromosomes (57). A concern with any RSV vaccine would be ensuring the right immune response was obtained. Several groups have tested of DNA vaccines for other viruses (84, 114) and further efforts into finding a safe and effective vaccine for RSV may include research into DNA vaccination.

Treatment

Passive antibody protection

There are two commercially available forms of immunoglobulin available for treatment of RSV, IVIG and RSV-IG. The latter is produced by screening plasma donors for high titres of RSV neutralising antibodies and purifying the Ig from these (60). In 1996, after encouraging results from trials, Respigram (RSV-IG) was licensed for use in the United States. Studies using this preventative approach are described below.

Premature infants with and without bronchopulmonary dysplasia (BPD)

Two trials were conducted, one by the National institute of Health (NIH) and the other by the PREVENT study group (50, 96). The NIH trial was blindly randomised and two dose levels were tested. The PREVENT trial was larger, placebo controlled, blindly randomised with one dose regime. Recruitment into the PREVENT trial stipulated that the infant required oxygen within the six months prior to admission onto the trial. Both studies showed there to be a reduction in RSV related hospitalisations, a decrease in the time spent in hospital, a decrease in the occurrence of otitis media, and the NIH trial showed a dose response relationship. The was a decrease noticed in the amount of time spent in intensive care, but this was not statistically significant due to the small numbers involved (50, 60, 96).

RSV -IG in infants and children with congenital cardiac disease

Two studies were conducted, one by the NIH and the other by the cardiac group (96, 219). Both of these studies had no placebo controls and the results from both were not statistically significant, although RSV disease was prevented more effectively in infants less than six months old. Certain safety concerns were raised from the trial which resulted in the recommendation that children with cyanotic congenital heart disease should not be given RSV-IG infusions (60). Drawbacks associated with this regime were that large intravenous doses had to be given to the infant monthly during the RSV season, with the main side effect seen being fluid overload. The treatment is also expensive.

It was speculated that some of the positive results seen from the trials were due to the education of the parents in other important aspects of reducing RSV infections in high risk infants. These would include no smoking around the infant, avoidance of infected adults, and frequent hand washing. Arrangements for child care may also be important, as an increased association with day-care facilities and RSV infections has been reported, even with elder siblings in childcare, presumably due to the excellent potential for viral transfer and spread (60). Wang and Law (1998) demonstrated that once lower respiratory symptoms of RSV infection have started the passive administration of RSV IG had no beneficial effects (243).

Monclonal antibodies for prevention of RSV infection

Monoclonal antibodies would have certain benefits over the use of RSV-IG, as they are less costly to produce and have no risk of viral contamination. Furthermore there is more viral neutralising ability per gram, which may reduce the volumes needed for protection (60). The most likely candidate for monoclonal antibody therapy is the fusion protein which exhibits the least variation between subtypes. As seen with the RSV-IG trials the method of delivery is important and it may be possible that a monoclonal antibody preparation could be given intramuscularly or even topically as nasal drops. The latter has shown promise in animal trials, and studies are ongoing (252).

Patient management

The supportive care has improved for the treatment of infants with RSV infections. Better patient management leads to fewer nosocomially transmitted infections, by early identification of RSV cases and droplet precautions (131). Bronchodilator therapy has given contradictory results using β-agonists and other forms of bronchodilators, and this approach is probably only of use in older children (242). Several studies have been conducted using corticosteriod therapy but they have shown very little benefit (60).

Ribavirin

Ribavirin is a synthetic nucleoside derivative of guanosine and *in vitro* shows activity against a wide range of viruses including RSV, measles, influenza A and B, parainfluenza, Lassa fever, bunyaviruses, hantaviruses, hepatitis A, B and C and HIV (60). It is quite toxic however, decreasing red blood cell production and concentrating itself within red blood cells (RBC), having a half-life similar to RBC. In the United States the FDA has licensed aerosolised ribavirin for treating infants with RSV. Interestingly, it has not been licensed for use in adults. Its effectiveness is controversial with many studies failing to give statistically significant results (60). Ribavirin probably causes a moderate reduction in RSV load. It is costly however and has a very cumbersome route of delivery, through a small particle aerosol generator.

Ribavirin in the immunocompromised

In the immunocomprimised ribavirin has been shown to be beneficial if given swiftly upon diagnosis of RSV. IVIG has shown to be beneficial in immunocompromised animal studies, in combination with ribavirin. A large dose of RSV-IG may prove to be more beneficial than several doses of IVIG because it is more concentrated (60).

Surfactant protein-A

Surfactant protein-A is part of the collectin family of human proteins, which are thought to be involved in innate immunity, as a defence against various viral and bacterial pathogens (150). The c-type collectins (of which surfactant-A is a member) are thought to bind to the carbohydrate surfaces of many micro-organisms and mediate phagocytosis. As surfactant protein-A is produced primarily by cells within the lung, and has been shown to bind a variety of bacteria and viruses, including influenza A, its anti-RSV activity has been studied (150). Kerr and Paton (1999) have shown that surfactant protein-A concentrations are altered in severe RSV infection (136). An *in vivo* model has also demonstrated the role of surfactant protein-A in RSV infection, enhancing the clearance of RSV (150). Future studies with it may include the development of it as a therapeutic agent for severe RSV infection.

Humanising antibodies

Future therapy for RSV infection is also under evaluation. Humanising mouse antibodies with a specific target of RSV F peptide have already been undertaken and have met with varying success (6, 169). Meissner *et al* (1999) tested a humanised antibody against the F protein of RSV (SB209763). High risk infants were given intramuscular injections of the antibody preparation, however no difference in RSV infection ratio was seen between the test and placebo groups. The dosing regime was suggested to be too low, at a maximum of 10 mg per kilogram body weight, and further studies of this antibody at a higher dose may be done (169). Another humanised antibody (MEDI-493) performed very well in clinical trials (6). Here intramuscular injections of 15 mg per kilogram body weight were given monthly to high risk infants. Large reductions were seen in hospital entry and duration of

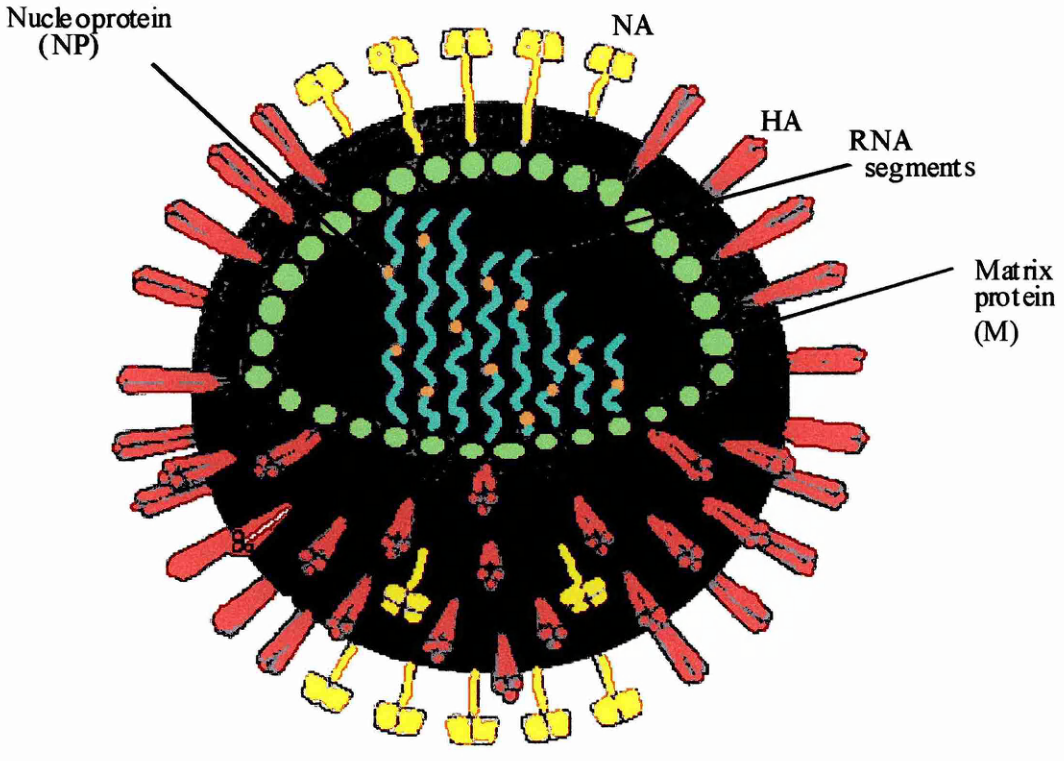
stay with the test group compared with the placebo. The humanised antibody appeared safe and was well tolerated (6).

RSV has been shown to be a severe cause of respiratory illness in specific subsets of the population, namely infants, the hospitalised and the elderly. Seasonal activity of RSV is seen during the winter months in temperate climates. Little to no information is known about the circulation of RSV in the general community, especially in the adult population.

Influenza

Influenza is a RNA virus belonging to the family orthomyxoviridae and the genus influenza. The virion has an envelope derived from the cell membrane with various virus protein projections. The virions are spherical or pleomorphic ranging between 80-120 nm diameter (174). The genome consists of seven or eight segments of negative sense RNA, totalling around 13,600 bp in length. There are three types of influenza, A, B and C, which are classified according to the serological properties of the internal proteins, the nucleoprotein (NP) and matrix (M). Influenza A and B have 8 segments whilst influenza C has only seven (144). Influenza A can be further classified according to the serological properties of two external proteins, the haemagglutinin (HA) and the neuraminidase (NA). There are fifteen different HA types (H1-15) and nine different NA types (N1-9) (208, 258). The natural host of influenza B and C is man, whereas the natural host for influenza A viruses is aquatic birds. In humans only influenza types A and B are known to cause severe disease, with influenza C causing mild illness. The haemagglutinninn and neuraminidase are found on the outside of the virus coat and are the major antigenic determinants of the virus (fig 1.5). The largest RNA segments code for one protein each (PB2, PB1, PA, HA), the rest encode additional structural and non-structural proteins depending on the virus subtype.

Figure 1.5 Diagrammatic representation of the influenza virus



Replication

Transcription takes place in the host cell nucleus. Influenza is one of only a few non-oncogenic RNA viruses known to conduct transcription in the host cell nucleus. The virus enters the cell by receptor mediated endocytosis and once inside the cell the virion binds to an endosome. The low pH of the endosome triggers the HA to fuse to the membrane of the endosome, and the ribonuclear proteins (RNP) are released reviewed by Krug, 1989 (140). It is believed that the intact RNP enters the nucleus through a nuclear pore. The synthesis of viral mRNA requires host cell primers. These are derived from the capped host-cell RNA polymerase II transcripts. This is achieved by a viral cap-dependent endonuclease which cleaves the cellular RNAs 10-13 nucleotides from their 5' ends. This process is known as "cap stealing" reviewed in Fields, 1996 (144). Replication of the influenza genome also requires an RNA dependent RNA polymerase activity, which is specified by

the virus. This enzyme lacks a 5' to 3' exonuclease activity (proof reading ability) and therefore has limited ability to correct mistakes during replication. As a result, there is a high frequency of mutations in any newly replicated population of influenza viruses. Mutations which confer a selective advantage result in rapid expansion of the population of variant viruses, reflecting the plasticity of RNA virus genomes (65). Accumulation of mutations leads to progressive genetic drift, and eventually confers sufficient changes for the virus to evade humoral antibody responses and re-infect, a phenomenon known as *antigenic drift*. The segmented nature of the influenza virus genome also allows the reassortment of virus segments. If a single cell is infected by two viruses simultaneously, mixing of the segments of RNA from different hosts allows the emergence of viruses with novel constellations of surface and internal proteins, a process known as *antigenic shift*.

Once mRNA is synthesised and translated the virion proteins are transported to the plasma membrane and the HA and NA are inserted into the membrane. The HA appears to be spread all over the surface of the membrane but the NA seems to appear in patches (140). All eight segments of the virus genome must be included in the viral nucleocapsid which then buds from the infected cell, thus acquiring a coat.

Genetic Diversity

Thus, genetic diversity amongst influenza A viruses is maintained by an intrinsically high mutation rate, coupled with the potential for segment reassortment and a number of host species, as described below. Influenza B and C have intrinsically lower rates of mutation, and a single animal reservoir and do not have multiple subtypes of HA and NA. The rate of mutation of avian influenza A virus genes in avian species, the natural host, is more similar to the rate of mutation observed in influenza B in the human host (53, 65). This is taken to reflect the adaptation of the virus to the natural host and the co-evolution of virulence determinants and host response to infection.

Haemagglutinin

The haemagglutinin (HA), originally named because it has the ability to agglutinate red blood cells, makes up a large portion of the envelope spikes (144). It is synthesised as a single polypeptide chain, which is cleaved into two disulphide linked chains, HA1 and HA2. This cleavage is essential for virus infectivity and is performed by host proteases (148). The HA is coded on RNA segment four. The HA is a trimer with a cylinder like structure, which has two major components: a long fibrous stem with a globular domain on top (259). The receptor binding pocket is located on the distal part of the HA and is highly conserved between isolates (259). The HA binds to sialic acid residues on the cell surface, from here it is believed the virus enters the cell by endocytosis and fuses with endosomal membranes under low pH conditions (255). This requires major conformational changes in the HA molecule. Once the virus particle has been endocytosed into the cell the HA fuses with the endosomal membrane allowing the release of the viral core. The HA is the main antigenic determinant of the virus.

Neuraminidase

The neuramindase (NA) protein, which acts like a sialidase enzyme, is less abundant on the surface of the virus than the HA, and is the second major antigenic determinant of the virus (144). The NA is found on RNA segment six and has a single open reading frame of 1362 nucleotides. The NA is a tetramer, with a mushroom like structure and a box shape head (147). The main function of the protein is thought to be the removal of sialic acid residues from newly synthesised viral glycoproteins, allowing newly formed particles to bud from infected cells and prevent aggregation. Virus mutants defective in NA function are seen to clump at the surface of infected cells instead of being released, and are unable to complete the replication cycle (187). NA is also involved in the cleavage of the α-ketosidic linkage between a terminal sialic acid and an adjacent sugar residue which may help to allow the virus to pass through the mucus in the respiratory tract (44).

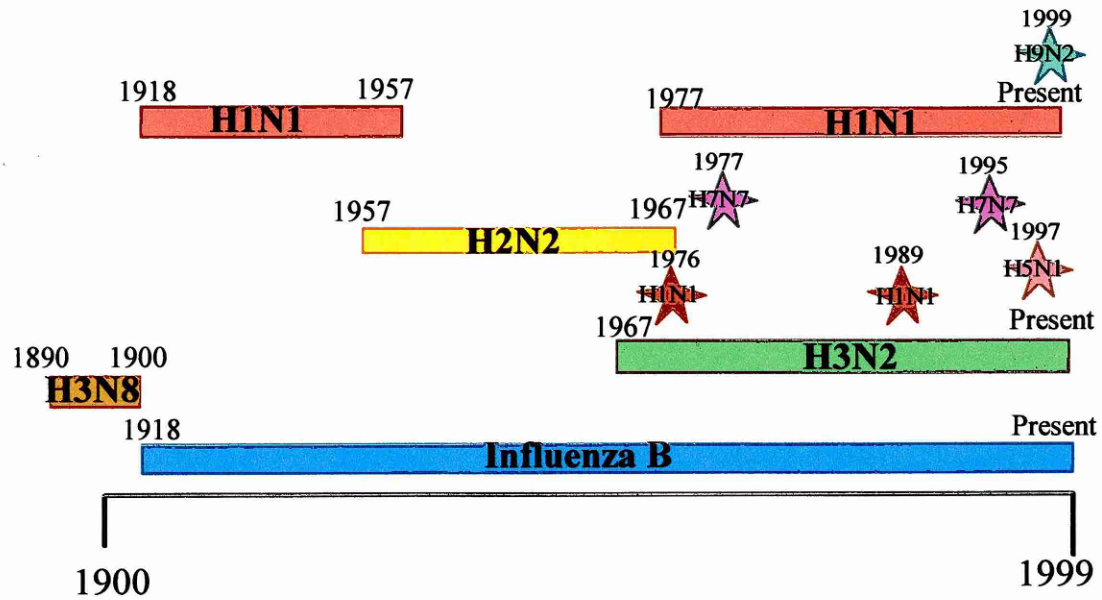
Epidemic influenza

For an influenza season to be classed as an epidemic certain criteria must be met. These criteria have recently been revised (199). Essentially, defining an epidemic requires knowledge of which influenza subtype is circulating in the population. Also, how many emergency house calls a GP has made, and reports from GPs of influenza and influenza like illness (ILI) must be considered.

Pandemic Influenza

One of the puzzles of influenza epidemiology is the appearance and disappearance of strains of influenza, occasionally in a cyclical manner (fig 1.5). During the course of the 20th century, a number of novel influenza viruses have emerged to cause pandemics. In 1918, 1957 and 1968, H1N1, H2N2 and H3N2 viruses respectively appeared, although the precise origin of these influenza strains remains to be clarified. In 1918, influenza A H1N1 was responsible for greater than 20 million deaths world-wide, killing twice as many people as the first world war. This virus killed people of all ages, unlike most winter seasons, where excess deaths due to influenza occur primarily in the over 65 age group.

Figure 1.5 Cycling of influenza subtypes from 1900 to 1999



The stars represent sporadic cases of influenza

The rapid spread of influenza virus coupled with the possibility of genetic reassortment and an extensive animal reservoir of infection ensures that the threat of a pandemic due to influenza is as great at the turn of the 20th century as it was at the beginning of the century.

Antigenic shift

It is believed that all three pandemics were caused by an antigenic shift in the virus. This means that the RNA segments coding for the genes for HA and/or NA were 'swapped' for those of a different strain. This can occur with animal strains of the virus, for instance, with the pig acting as a mixing vessel for avian and human strains of the virus (251). Until recently the 1918 pandemic was believed to be caused by a human/swine reassortant with, unusually, peak activity during the summer months. Reid *et al* (1999) have recently characterised the HA gene of the 1918 strain of influenza (201). Paraffin embedded lung tissue and samples of lung taken from victims buried in permafrost were examined and the HA of this virus characterised to be of H1N1 subtype (201). Comparison of this deadly strain revealed that although it was closely related to avian strains, it was mammalian and had probably been adapting in humans for some time (201).

Antigenic drift

The influenza virus also varies annually by the phenomenon antigenic drift. This is the accumulation of mutations within the viral RNA genome that alters the antigenic properties of the virus, allowing it to escape the host immune system. As the influenza virus has this ability of antigenic shift and antigenic drift, constant surveillance of circulating strains within the population is necessary so the vaccine can be updated annually.

Natural reservoirs of influenza viruses

Influenza virus infects and replicates in epithelial cells in the respiratory tract in humans and causes respiratory illness. The influenza A viruses also infect a number of other mammalian species besides humans, including horses, swine and a variety of sea mammals (249). Only two of the 15 different HA subtypes of influenza A (H1N1 and H3N2) are commonly found in humans today. However all 15 HA subtypes of influenza A have been found in aquatic birds, and most of these types are associated with asymptomatic infection,

although two types (H5 and H7) can cause severe infection in domestic poultry. In avians influenza A is a disease of the enteric tract and is transmitted via the faecal oral route. It is thought that migratory birds are important in disseminating the spread of different subtypes of influenza, particularly where there is congregation of birds on migratory flyways. Influenza A has been recovered from unconcentrated lake water. Shedding of virus particles which are relatively stable in a moist environment ensures the successful transmission of infection between generations and species of birds. Infections spill over into the domestic bird population (ducks, geese, chickens, turkeys) especially where there is mixing of feral and domestic birds.

Receptor binding

Human influenza A HA types preferentially bind to sialic acid via a α-2-6 link to galactosidase residues whereas avian influenza types preferentially bind to α-2-3 linked sialic acid (207). As a consequence of this preferential binding, it was considered that direct avian to human transmission of influenza virus was unlikely. Domestic swine have both α-2-6 and α-2-3 linked sialic acid in their respiratory tract and is thought that a dual infection of swine by influenza viruses from different species provides an opportunity for exchange of RNA segments and emergence of an influenza virus capable of infecting and rapidly spreading between humans (119). However, in Hong Kong in 1997 a highly pathogenic avian influenza virus, H5N1, directly infected several humans, causing severe illness and six deaths (217). The source of the H5N1 outbreak in 1997 was infected poultry in live bird markets in Hong Kong and more widespread dissemination was prevented by a cull of approximately 1.5 million chickens. Although this particular virus was not proven to be transmissible from human to human, the potential threat of the avian reservoir of influenza subtypes to human health was clearly demonstrated. Other sporadic cases of animal to human transmissions of unusual influenza A subtypes have occurred. As the surveillance of influenza improves it is likely that more such events will be identified.

Clinical presentation

Human influenza is spread primarily by the aerosol route or by direct contact. With a short incubation time of 1-4 days and abrupt onset of illness, epidemics can start explosively and on a large scale (55). An adult patient with an influenza infection can have many symptoms, most commonly temperature, muscle aches, and fatigue. Fever can last between 3 and 6 days at which point the respiratory symptoms may become more marked. Illness can last up two weeks and may need hospitalisation in severe cases. In children the clinical symptoms can be similar to adults, but fever is higher and there is an increased risk of otitis media. In children of less than 3 years old there may be more pronounced systemic features, particularly abdominal pain and vomiting (181). Although the factors which influence the symptoms of an influenza infection are not fully understood, it is most likely that symptoms are due to a combination of virally induced cytopathic effects and the host immune response to the virus, involving the secretion of cytokines. In particular, IFN-α and IL-6 are associated with the early phases of influenza infection (106).

Morbidity associated with influenza epidemics

Influenza is a seasonal disease in the Northern Hemisphere, occurring between October and March every year, although the exact timing, duration, and magnitude of epidemics varies year to year. Complications associated with influenza infections occur in both the upper (URT) and lower (LRT) respiratory tract. Otitis media is a common complication in children, and conjunctivitis occurs in both adults and children. Primary viral pneumonia, or more commonly combined viral and bacterial pneumonia can occur following influenza which has spread to the LRT (181).

Every year the total number of deaths from all causes peaks during times of circulation of influenza viruses. In an average H3N2 year, the number of deaths due to influenza in the UK is estimated to be between 10,000 to 13,000 (78). It has also been estimated that the number of deaths year to year during the interpandemic periods will cumulatively exceed the number of deaths due to influenza in the three pandemic periods (78).

Diagnosis of influenza

Until recently, diagnosis of influenza has relied on traditional methods of cell culture, which are still crucial for assessing the antigenic properties of circulating strains. There are now many diagnostic methods that are used for the identification of influenza. The virus can be readily cultured in a variety of cell lines and embryonated chicken eggs in the laboratory. Testing of cultured virus using panels of antisera (usually obtained from ferrets) allows extended typing of the virus by strain designation and comparison of similarity to past circulating virus. This technique is considered to be the "gold standard" for influenza detection but can, however, be a lengthy process often taking a week or longer. Haemaglutinnin inhibition testing utilises the haemagluutination properties of the virus. If no virus is present to aggregate the red blood cells they settle as a uniform dot at the bottom of the test vessel. However if virus is present then it will clump the red blood cells together and give a diffuse appearance in the base of the test vessel.

More recent diagnostic methods, including molecular diagnosis using PCR, can now contribute to immediate patient management (Table 1.2) and may be very helpful in outbreak situations (4). PCR or ELISA, which take on average 2 days to perform, require more specialist equipment and are more expensive to conduct than virus culture (70). Additionally, there are several near patient tests either already marketed or about to be launched which may impact on the speed of diagnosis of influenza in different settings, although their utility in the UK has not yet been assessed. These tests can be completed in 15 minutes or less, and include Directogen from Becton Dickinson, Microbiology Systems.

Table 1.2 Diagnostic methods for influenza detection

Method	Sample required	Estimate Cost (per sample)	Time Taken	Advantages	Disadvantages
Culture	Nasopharyngeal aspirate, Nose and throat Swab, Bronchoalveolar lavage	£10-20	3-7 Days	Whole virus measured Virus recoverable "Gold Standard"	Requires infectious virus Requires several cell lines / embryonated hens eggs Highly skilled
IF	Nasopharyngeal aspirate, Nose and throat Swab, Bronchoalveolar lavage	£5	2 Hours - 1 Day	Rapid	Requires intact cells, Highly skilled No virus recoverable Specialised equipment needed Labour intensive, Non-specific Numbers limited
Antigen	Nasopharyngeal aspirate, Nose and throat Swab, Bronchoalveolar lavage	£5-10	15 mins - 1 Day	No specialised facility needed Can be "near patient" testing Low skill Rapid	No virus recoverable Poor sensitivity/specificity? Often non-proven technology
PCR	Nasopharyngeal aspirate, Nose and throat Swab, Bronchoalveolar lavage, unfixed post-mortem tissue	£20-30	1-2 days	Sensitive Allows further molecular analysis	No virus recoverable Specialised equipment / laboratory needed Highly skilled
Serology					
HI*	Serum	£5	2# Days	Sensitive and specific	Retrospective, paired samples needed
CFT**	Serum	£5	2 Days		Insensitive, retrospective, paired samples needed

* Haemaglutination inhibiton # Acute and convalescent sera reqired (14 days) ** Complement fixation test

Prevention and Treatment

The main strategy for controlling influenza has focused on vaccination, using trivalent inactivated vaccines. Every year circulating influenza strains are monitored by the World Health Organisation (WHO) global influenza surveillance network and the information used to decide which strains should be incorporated into the vaccine for the following winter season. The vaccine consists of influenza A H1N1 and H3N2 and influenza B virus antigens, and is made from viruses grown in embryonated eggs. Owing to the time required to make the vaccine (currently 6-9 months), the decision of which strains to incorporate into the vaccine has to be made months before the influenza season starts (54). This can sometimes result in a poor match between the vaccine and circulating strains for the next seasons circulating strains. The effectiveness of the vaccine depends on how close the vaccine strains matches strains circulating in the population, as well as on the age and immunocompetence of the recipient. The vaccine efficacy when vaccine is well matched to circulating strains is estimated at 70-90% in healthy adults less than 65 years old, and 30-80% in the elderly and chronically ill (1, 118). There is now a large body of evidence which demonstrates both clinical efficacy and cost benefit of influenza vaccination, particularly in the elderly population (178, 179). This has provided the evidence underlying the recommendation in 1998 that all over 75s in the UK should receive influenza vaccine irrespective of risk (62). The current guidelines for vaccination in the UK also include any person over 6 months, who because of age or medical condition, are at increased risk from influenza infection (62).

Influenza vaccine is administered to any person requesting it. However despite efforts at targeting the vaccine, it is clear that individuals in key groups are not receiving vaccines. Over a three year period of monitoring vaccine uptake in England and Wales, 38% of people in a high risk category did not receive vaccine in any of the three years, whereas 23% of persons in a no risk category received vaccine in all three years (118) (Personal communication, C. Joseph). The reasons for this are not clear, but may include under recognition by medical staff of the impact of influenza and the need for vaccination

Novel vaccine strategies

Several novel techniques are being explored in an attempt to produce new vaccines, including purified haemagglutinin (HA) vaccines, DNA vaccines (250) and live attenuated vaccines (13).

Purified haemaglutinnin vaccines

These vaccines circumvent the current reliance on chicken eggs. Recombinant HA may be synthesised from a variety of sources including baculovirus or *E. coli* expression systems. This is of obvious benefit to people with egg allergy and may allow more rapid vaccine production. Vaccine strains of influenza grown in cell culture are also less likely to cause egg allergy (250).

DNA vaccines

Direct inoculation of DNA has caused great interest as a means of vaccination since proof-of-principle studies showed its potential in the early 1990s (250). The technique is based on the observation that direct intramuscular injection of DNA results in expression of genes encoded by the DNA. The basic method has several inherent advantages. These are the stability of DNA relative to other protein based vaccines, no anti-vector immune response, no egg involvement, and an apparent MHC class I presentation which is ideal for cytotoxic T cell response. Efficacy of DNA vaccination against influenza has been demonstrated in animals (86), although safety concerns will temper the use in of it humans for some time.

Live-attenuated intranasal vaccine

An intranasal cold-adapted vaccine is in late clinical development in several countries (13). For this the ability of influenza A to reassort is exploited. A strain that has been attenuated by cold-adaptation is used as the donor virus, to which is added an appropriate wild-type variant that contains the NA and HA segments against which protection is sought. This mixture is cultured together so that the resultant vaccine strain contains an attenuated genome but expresses the wild-type HA and NA surface antigens. The vaccine strain maintains its attenuated phenotype and genotype through passage. Live attenuated vaccines will allow the induction of mucosal immunity similar to that induced by natural infection

with wild virus, the induction of a significant cytotoxic T-lymphocyte response, superior protection from influenza in the upper respiratory tract, limiting viral spread within the population, and a very much easier, convenient and acceptable method of vaccination. The best results obtained with live attenuated vaccines appear to be with children at the present time (13).

Antivirals

Until 1999, there were two drugs licensed for use against influenza, amantadine and its methylated derivative, rimantadine, although these have not been widely used. These drugs work by blocking an early stage of virus replication, and act on the influenza A virus M2 (105). M2 is not a constituent of influenza B and these drugs therefore lack any efficacy against influenza B virus. Although not in widespread use in the UK, amantadine has been used elsewhere in the world, and is effective as a prophylactic treatment and also as a therapeutic agent. It does, however, have a significant number of side effects including gastrointestinal complaints, sleep disturbance and difficulty in concentrating. Elimination of the amantadine is related to renal function, whereas rimantadine is metabolised by the liver and causes fewer side effects.

New treatment options for influenza: neuraminidase inhibitors

The crystal structure of NA was solved in 1983 (237), which laid the foundations for the computer-aided design of inhibitors of the NA reported in 1993 (146). This work was crucial to the development of the NA inhibitors (NI). One NI, zanamivir (Glaxo Wellcome), has already been licensed in the UK in 1999, but not available on the National Health Service (NHS) as yet, and a second drug, GS 4104 (Roche) is expected to follow closely behind. The two drugs both target the NA, and both act at the active site of the molecule. Zanamivir is given intranasally and GS4104 is given orally (21).

Neuraminidase inhibitors and resistance issues

As with nearly all antiviral agents, one of the problems with anti-influenza agents is the development of strains that are resistant to the drug, and retain full pathogenicity. Clinical use of amantadine and rimantadine has been tempered by these problems and they have tended to be used in a limited number of settings. Resistance to amantadine and rimantadine was reported very early on in the development of them with resistant isolates appearing after only one passage in some animal models. The observation that the active site of the enzyme was highly conserved across all types of influenza A and B may have implications for whether or not natural infections may give rise to clinically relevant drug-resistant influenza strains. Resistance to zanamivir and to GS 4104 does not appear to occur as readily as has been seen with amantadine and rimantadine, although the true picture of resistance will not emerge until these drugs are used on a wide scale (190).

Resistance mechanisms

The evidence that has emerged so far on the use of zanamivir, indicates that no clinically significant zanamivir-resistant strains have been detected during acute treatment in otherwise healthy individuals (21). However mutations in the NA may occur and account for relative resistance to zanamivir *in vitro*. Viruses containing mutations of the NA show attenuated sensitivity to zanamivir *in vitro* and, to a lesser extent, in animal models. However, strong evidence is now emerging that viruses that have undergone these mutations are in some way functionally impaired. As zanamivir binds to the NA active site, it implies that mutations that have any effect on the affinity of NA for zanamivir will necessarily affect the affinity of NA for its natural substrate, sialic acid, and that this will have an attenuating effect on the virus (21, 190).

CHAPTER 2

General Materials & Methods

Virus stocks

RSV A and B strains (Long (A), B908106, B3/60 and 18537) were grown in Hep-2 cells obtained from the European cell culture collection (ECACC, Porton Down, Salisbury UK). Virus was harvested when 70% of the monolayer displayed a cytopathic effect (CPE), usually 2-4 days post infection. Virus stocks consisted of a mixture of mechanically disrupted cells and supernatant, and were stored at –70°C immediately after harvest until use. Influenza A (H3N2 and H1N1) and B strains were grown in the allantoic cavity of embryonated eggs or in Madin Darby canine kidney (MDCK) cells in the presence of tolylsulfonyl phenylalanyl chloromethyl ketone (TPCK) trypsin. Virus containing tissue culture supernatant or allantoic fluid was stored at –70°C until required.

Several RSV B strains (see Table 3.1) were kindly supplied by Dr. Pat Cane of Birmingham University and were grown in Hep-2 cells. Virus was innoculated on to a 70% confluent monolayer of Hep-2 cells and left at 37°C, and monitored daily. The viruses were passaged when the viral CPE scored 3-4 (65-75% of cells infected). Ballotini were used to break the cell monolayer prior to passaging.

Clinical specimens

Nasopharyngeal aspirates, broncheoalveolar lavages, endotracheal aspirates and untyped isolates of RSV were obtained from hospitals in the London area from clinical diagnostic material collected between October 1995 and May 1996. Combined nose and throat swabs were obtained from cases of community respiratory illness investigated during clinical virological surveillance of influenza in England and Wales in the 1995/1996, 1996/97 and 1997/98 winter season. All clinical material was stored at –70°C until use. The combined nose and throat swabs were made of rayon material. There is no evidence that rayon is inhibitory to PCR.

Titration of virus infectivity

RSV: Hep-2 cells were seeded at $5x10^4$ cells/ml in minimal essential medium (MEM) (Gibco-BRL) containing 10% fetal calf serum in 96 well microtitre plates (Greiner Labortechnik) and incubated overnight at 37°C in a CO_2 atmosphere. One hundred µl of each virus dilution were added to wells containing cell monolayers in duplicate. The plates were sealed and spun at 1,500g for 45 min at 37°C. Samples were aspirated from the wells and replaced with MEM medium containing 2% fetal calf serum and further incubated for 24 hrs at 37°C in 5% CO_2. Media was expelled from the plates and the cells washed with phosphate buffered saline (PBS) prior to fixation for 20 min with absolute methanol / 2% (vol/vol) hydrogen peroxide.

Virus infected cells were detected by the addition of polyclonal goat anti-RSV antibody (Biogenesis, UK) diluted 1:800 in PBS / 0.05% Tween 20 (PBST) for 1 h. Subtyping information was provided by a RSV B specific monoclonal antibody (Clone number 7858, National Bacteriological Laboratory, Sweden) diluted 1:1000 in PBST in parallel infected wells. Following three washes with PBST, rabbit anti-goat or anti-mouse horseradish peroxidase (HRP) conjugate (Chemicon) was added to each well and the plates incubated for a further hr at 37°C. After washing three times in PBST, freshly prepared insoluble HRP substrate AEC (3 amino-ethyl-carbazole) was added to each well and incubated at room temperature for 45 min in the dark prior to reading the cell ELISA. Wells were examined under a light microscope for the presence of pink stained cells. Each pink cell was considered to represent infection by a plaque forming unit of RSV.

Influenza: Confluent MDCK cells were washed with PBS and incubated for one hr at room temperature with virus inoculum diluted in MEM. The inoculum was removed, and the cells overlaid with medium containing 0.5% indubiose, nonessential amino acids, and 3 mg of TPCK treated trypsin / ml and incubated at 37°C in 5% CO_2 for 48 h. The cells were fixed with 5% (vol/vol) glutaraldehyde and stained with 2% vol/vol carbol fuschin (70, 232). The majority of infectivity assays were performed by other members of the Respiratory Virus Unit.

PCR anti-contamination precautions

All PCR work was carried out under conditions designed to prevent the occurrence of carry over contamination. Nucleic acid extraction, PCR reaction mix setup, thermocyling and gel analysis were all conducted in separate rooms. Transfer of the primary product to the secondary reaction mix was done in a PCR workstation (LabSystems) which was equipped with a UV light. Each room has dedicated equipment and clothing, and all pipette tips used were plugged to prevent any possibility of contamination. All work was carried forwards from RNA extraction through to gel analysis of the final PCR amplicon.

Nucleic acid extraction and cDNA synthesis

RNA was extracted from 100-150 µl volume of sample (egg fluids, tissue culture material, clinical specimens and water controls) with a guanidinium thiocyanate-silica binding method (15). Specimen was added to a tube containing 840-890 µl of lysis buffer (1M guanidinium thiocyanate, 0.1 M Tris-HCl, pH 6.4, 0.2 M EDTA pH 8.0, 2.6 g Triton X-100) and 10 µl of silica suspension, mixed, incubated for 10 min at room temperature and the RNA bound to the silica washed twice with 1 ml buffer L2 (1M GuSCN, 100 ml 0.1 M Tris-HCl pH 6.4), twice with 1 ml of 70% (vol/vol) ethanol, once with 1 ml acetone and then dried at 56°C for 10 min. RNA was then eluted with 30 µl of RNase free water and converted into cDNA by RT-PCR. For reverse transcription 22.2 µl of RNA were added to a reaction (17.8 µl) containing 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 7.5 mM $MgCl_2$, 1.5 mM of each dNTP, 25 ng of random primer pdN6 (Pharmacia), 1.6 U of RNasin (Promega), and 200 U Moloney murine leukemia virus reverse transcriptase (Gibco BRL). The reaction was incubated at room temperature for 10 min, 37°C for 45 min, 95°C for 5 min and quenched on ice (15, 92).

Multiplex primer design

The RSV primers were designed from sequences in the GenBank and EMBL databases. OLIGO primer analysis software version 5 (National Biosciences Inc.) was used to select primers with similar Tm and GC content. The primers were then assessed with this software for any possibility of mispriming on any other viral templates. The primers were

designed to produce amplicons of molecular mobilities that would be easily distinguished from each other on agarose gels.

Multiplex PCR optimisation

All optimisation steps were performed on individual viral RNA templates in succession, with only one parameter altered at a time. Experiments varying the annealing temperatures of a PCR reaction were performed on a Robocyler (Stratagene). A range of buffer systems were tested (see below). Two non-mechanical means of Hot Start were tested, *Taq* polymerase Hot Start Antibody (Clontech) and *Taq*Gold (Perkin Elmer) according to the manufacturers instructions.

Multiplex PCR

The primers used are shown in Table 3.1. Each primer pair was used at 5 pmol in the primary amplification and 25 pmol in the secondary amplification reaction. For the primary PCR, 20 µl of cDNA was added to 80 µl of reaction mix containing 10 mM Tris-HCl pH8.8, 3.5 mM $MgCl_2$, 2.5 mM KCl and 1.5 U *Taq* polymerase. Amplification, using a DNA Engine thermocycler (MJ Research), consisted of 1 cycle at 94°C for 2 min, followed by 35 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 1 min. Two µl of primary product were then transferred into 48 µl of secondary amplification mix (as above with 0.2 mM each dNTP). The samples were then incubated for one cycle at 94°C for 2 min, then 35 cycles of 94°C for 1 min, 60 °C for 1 min, and 72°C for 1 min.

Agarose gel electrophoresis

For multiplex PCR amplicon analysis 15 µl of PCR product were mixed with 2 µl Orange G loading buffer: 0.25% Orange G which was 10% Ficoll in TE (10mM Tris, pH 7.4, 1mM EDTA) and run on a 2.25% agarose gel (NuSieve 3:1, FMC BioProducts) in 1x TBE (89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH8.4). For all other amplicon analysis 15 µl of PCR product were mixed with 2 µl Orange G loading buffer as above and run on a 1% agarose gel (Seakem FMC BioProducts) in 1x TBE. The amplicons were visualised using ethidium bromide staining and photographed using a Polariod camera.

RCGP sample analysis

Combined nose and throat swabs were collected from patients presenting with influenza or influenza like illness by their GP. The parameters for definition of influenza or ILI were not defined (80). Samples were sent by post into the respiratory virus unit at Colindale for analysis (79). This often produced a delay of 2 days between sample collection and receipt. Upon arrival the samples were vortexed (from 96 winter season onwards).

95/96 and 96/97 season:

Samples were extracted and tested for influenza first and cDNA stored at -20°C until tested by multiplex PCR. The majority of extractions were performed by other members of the respiratory virus unit. The samples were tested in batches of no more than 20 with negative controls placed after every 5 samples. Positive controls were of medium to low titre (dilution $10^{-3/4}$) and were at the end of every run. Re-testing of samples involved re-extraction form the initial starting sample.

97/89 season:

These samples were tested as for the previous seasons, except all samples were freshly extracted before testing by multiplex PCR as stored cDNA was unavailable. The majority of extractions and amplification reactions were performed by Chris Cave, of the respiratory virus unit.

Ammonium chloride buffer conditions for PCR

Reactions were performed with 1X Qiagen buffer, 1 x Q-Solution, 3.5 mM $MgCl_2$, and 1.5 U *Taq* polymerase in both rounds of the nested reaction. The primary amplification primers were used at 5 pmol, and the secondary amplification primers at 25 pmol. For the primary PCR 20 µl of cDNA was added to 80 µl of reaction mix. Amplification, using a DNA Engine thermocycler (MJ Research), consisted of 1 cycle at 94°C for 2 min, followed by 35 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 1 min. Two µl of primary product were then transferred into 48 µl of secondary amplification mix. The samples were then incubated for one cycle at 94°C for 2 min, then 35 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min. Amplicons were visualised by ethidium bromide staining following electrophoresis on 2.25% NuSieve (FMC BioProducts) agarose gels.

Optimisation of the PCR with the Stratagene Optiprime kit

Five µl of each buffer labelled 1 - 12 (Table 2.1) were placed into correspondingly labelled tubes. For the first round reaction the follwing master reaction mix was made for each template to be optimised; 12.5 µl of Optiprime master mix, 12.5 µl dNTP mix (0.2 mM of each dNTP), 12.5 µl of the primers (Table 2.1), 125 µl cDNA, 1.5U *Taq* polymerase. Forty five µl of reaction mix was placed into each buffer and amplified. Amplification, using a DNA Engine thermocycler (MJ Research), consisted of 1 cycle at 94°C for 2 min, followed by 35 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 1 min. Two µl of primary product were transfered into 5 µl each corresponding Optiprime buffer, with 43 µl of master mix (as above excluding the cDNA). Amplification consisted of one cycle at 94°C for 2 min, then 35 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min. Amplicons were visualised by ethidium bromide staining following electrophoresis on 2.25% NuSieve (FMC BioProducts) agarose gels.

Table 2.1 Composition of Statagene Optiprime Buffers

Buffer No.	Tris-HCl	$MgCl_2$	KCl
1	100 mM pH 8.3	15 mM	250 mM
2	100 mM pH 8.3	15 mM	750 mM
3	100 mM pH 8.3	35mM	250 mM
4	100 mM pH 8.3	35 mM	750 mM
5	100 mM pH 8.8	15 mM	250 mM
6	100 mM pH 8.8	15 mM	750 mM
7	100 mM pH 8.8	35 mM	250 mM
8	100 mM pH 8.8	35 mM	750 mM
9	100 mM pH 9.2	15 mM	250 mM
10	100 mM pH 9.2	15 mM	750 mM
11	100 mM pH 9.2	35 mM	250 mM
12	100 mM pH 9.2	35 mM	750 mM

Densitometry

Gel pictures were scanned and analysed on a Power Macintosh using the software Image Assist from GS Microtech. The scanned images were then cropped and inverted using RestritoScan software. Densitometry was then performed on the image using Scan Analysis software (Biosoft, Cambridge). Editing of densitometry plots was performed using ClarisWorks software.

Probe design

Probes were designed to each of the five amplicons using OLIGO and by eye. The probes were designed to have a similar Tm and possible mis-priming events were analysed using OLIGO 5. Probes were synthesised (MWG Biotech) with a 3 base pair non-complementary spacer region and biotin molecule at the 5' end (Table 3.6).

ELOSA (enzyme linked oligo-sorbant assay)

The secondary PCR reverse primers were synthesised with a fluorescein molecule bound at their 5' end (MWG Biotech). Probes were diluted in 0.01% PBST, and each well of a streptavidin coated microtitre plate (Labsystems) was coated with 100 ng of probe for 30 min at room temperature. The wells were then washed three times in 0.01% PBST. Twenty µl of PCR product was heated to 95°C for 2 min and diluted in 80 µl of hybridisation buffer (5 X SSC, 1% Boehringer Mannheim blocking reagent, 1% lauryl sarcosine) heated to 55°C, and place in the wells. The microtitre plate was incubated at 55°C for 30 min. The wells were then washed three times in 0.01% PBST. Anti-fluorescein-horse radish peroxidase conjugate (FITC-HRP Amersham) was diluted 1 in 1000 in hybridisation buffer and 100 µl added to each well, then incubated at room tempertature for 30 min. The plate was washed three times in 0.01% PBST and 100 µl of TMB (Europa Research Products Ltd.) added and incubated in the dark for 30 min at room temperature. The colour reaction was stopped by adding 50 µl of 2 M sulphuric acid, and the OD of each well measured at a dual wavelegth of 450/620 nm.

Lightcycler

Ten µl reactions were performed in LC capillaries (Biogene Ltd.).

Individual reaction

Individual reaction mixes contained 0.5 µl SYBR-green diluted 1 in 10,000, 1 µl of forward primer (5 µm concentration), and 1 µl of reverse primer (5 µm concentration), 5 µl of LightCycler Master Mix $MgCl_2$ buffer (Biogene Ltd.) and 2.5 µl of primary PCR product. The cycling conditions were 95°C for 0 sec, 55°C for 2 sec, 72 °C for 10 sec for 45 cycles (ramp times were 20°C per second).

Multiplex reaction

The multiplex reaction mixture contained 0.5 µl SYBR-green dilution 1 in 10,000, 1 µl of forward primer mix (all secondary sense primers, each primer had a final concentration 5µm), and 1 µl of reverse primer mix (all secondary reverse primers, each primer final concentration 5µm), 5 µl of LightCycler Master Mix $MgCl_2$ buffer (Biogene Ltd.) and 2.5 µl of primary PCR product. The cycling conditions were 95°C for 0 sec, 55°C for 10 sec, 72°C for 10 sec for 45 cycles (ramp times were 20°C per second).

Cycle by cycle fluorescence monitoring and melting point analysis was performed by the LC software.

RSV analysis techniques used at CPHL

RSV G primer design

Primers were designed both by eye and using OLIGO 5 primer design software. Primers were designed to amplify part of the first variable region of the G gene from both RSV A and B subtypes. Primers were designed to be as similar as possible in their physical and chemical characteristics.

RSV G PCR

A nested amplification was performed with primer concentrations of 5 pmol in the primary reactions and 25 pmol in the secondary round reactions. In the primary PCR 20 µl of cDNA were amplified in a 100 µl final volume containing PCR buffer (20 mM Tris-HCl pH 8.4,

50 mM KCl, 1.5 mM $MgCl_2$) 0.2 mM each dNTP and 1.5 U *Taq* polymerase. For the second round reaction 2 µl of the primary product were transferred into 48 µl of the above mix with 3 U of *Taq* polymerase instead of 1.5 U. Cycling conditions consisted of 2 min at 94°C, then 35 cycles of 94°C for 1 min, 50°C for 1 min, 72 °C for 1 min, for both rounds of amplification.

Gel purification

Gel fragments were excised from the gel using a clean scalpel. PCR products were purified from the agarose using QIAquick gel extraction columns (Qiagen) according to the manufacturers instructions.

Sequencing

RSV A and B PCR amplicons were sequenced using the secondary primers (Table 5.2). Sequencing was performed using dye deoxy terminator chemistry and cycle sequencing (Perkin Elmer) according to the manufacturers recommendations. Samples were run on an Applied Biosystems 373A sequencer.

Sequence analysis

Output files from the 373A sequencer were edited in the program Analysis using the Call bases option to remove all questionable data from the beginning and end of the sequence. Files were then imported into SeqEd (Applied Biosystems) for comparison of forward and reverse sequences. If many disagreements were seen between the two sequences (usually associated with high background and low signal intensity) the sequencing was repeated. Once a consensus sequence was generated the text sequence was transferred EditSeq (Lasergene, DNAStar). From this format the sequences were exported into MegAlign (Lasergene, DNAStar) where they were placed inframe and aligned using the Clustal algorithm. Sequences were trimmed to be of the same length prior to analysis (258bp).

Phylogenetic analysis

Sequences which were aligned in MegAlign (Lasergene, DNAStar) were exported in either a GCG or Paup format and converted into a Phylip format using the program Readseq. These files were the input files into the analysis packages Puzzle, DNADist and Seqboot. The transistion/transversion was calculated using Puzzle 4.0 (41-43).

Branch length determination

Maximum likelihood distance matrices were calculated using the programme DNADist. The output file from this was analysed by Fitch which produced a tree in a text format. The program Treeview was then used to view the tree (41-43). The trees produced were compared with trees produced using the maximum likelihood method implemented in the program Dnaml, before the boot strap value determination was conducted.

Boot strap value determination

One hundred new random datasets were created using Seqboot. The output file from this was input into DNADist to calculate the maximum likelihood distance matrices for each dataset. The output file from this was analysed in Neighbor or Fitch which produced a tree for each dataset in text format. The output file from this was input into the programme Consense to give the consensus or majority tree with bootstrap values in text format. Treeview was then used to view the tree and bootstrap values (41-43).

Phylogenetic trees

The branch length tree viewed in Treeview was saved as a graphics file and then input into ClarisWorks for editing. Boot strap values were then placed onto the relevant branches. Values over 70 are shown plus those of other important branches. The sequence names were changed for confidentiality based upon the scheme given below.

Naming of sequences

In order to retain patient confidentiality the names of the samples were changed. To aid interpretation of results a naming system was employed with the initial letter indicating subtype (either A or B). The next two numbers indicate the season which the sample was from, with 95 representing the 1995/96 season, 96 representing the 1996/97 season and 97

representing the 1997/98 season. The following number indicates the order, of those analysed here, in which the sample arrived in the laboratory. This also indicates the time the sample was taken during the season. Finally a lower case initial indicates whether the sample was from a child (c), adult (a) and associated with a dual infection (d). For these purposes a child was classed as 15 years old or younger. For example A967c would indicate a sample taken during the 1996/97 season, seventh in order of arrival to be analysed from this season, and the sample was from a child.

Synonymous and non-synonymous ratios (Ks/Kn)

Alignments were imported into the program BBedit for editing into a suitable format. Ks/Kn ratios were calculated using the program MEGA.

RSV analysis techniques used at CDC

The methods used at CDC were used as a comparison with the ones developed at CPHL (191).

Nucleic acid extraction

RNA STAT (guanidium isothiocyanate-phenol) was added to the specimen and then mixed with chloroform according to the manufacturer's instructions (Tel-Test Inc, Friendswood, Texas). Following centrifugation the lysate separates into two phases (aqueous and organic) with the RNA remaining in the aqueous phase. The RNA was then precipitated with isopropanol, washed with ethanol and solubilised in water.

Specific reverse transcription cDNA synthesis

Seven µl RNA was annealed with 20 mM F1 primer and 2 µl 5X buffer (250 mM Tris-HCl, 40 mM $MgCl_2$, 150 mM KCl, 5 mM dithiothreithol pH8.5; supplied by Boehringer Mannheim with AMV). This was mixed and placed in a 480 thermocycler (Perkin Elmer) for 5 min 95°C and then 52°C for 20 min. Then 5 µl 10X RT buffer (100 mM Tris-HCl pH 8.3, 6 mM $MgCl_2$, 10 mM DTT), 5 µl 10 mM dNTPs, 1 µl of RNase inhibitor (Boehringer Mannheim), 40 units AMV (Boehringer) and 27 µl SDW were added to each tube which was cycled in a 480 as follows: 42°C 60 min, 94°C 5 min, 4°C hold.

RSV G PCR

PCR was carried out with a mix of 5 µl cDNA, 10 µl buffer (150 mM Tris-HCl, 500 mM KCl, 15 mM $MgCl_2$, DMSO 3%, glycerol 8%), 20 mM dNTPs, 40 mM forward and reverse primer and 5 U *Taq* polymerase bought up to 100 µl with SDW. Cycling proceeded on either a GeneAmp 9700 or 2400 thermocycler (Perkin Elmer) as follows: 94°C 2 min, then 30 cycles of 94°C 1 min, 52°C 1 min, 72°C 2 min, with a 4°C hold.

The forward primer was GABSHORT (sequence unavailable) and the reverse primer F1 (191). Products were run on 1.2% agarose gels (Gibco Pure Agarose) strained with ethidium bromide and viewed under U.V. light.

ß-actin PCR

Primers for ß-actin (246) were used to amplify RNA from clinical specimens. RT-PCR was carried under the conditions described above.

CHAPTER 3

The design, optimisation and validation of a multiplex PCR for influenza A H1N1, H3N2, influenza B and RSV A &B

Introduction

PCR

Since the description of PCR as a useful laboratory technique in 1985 (210) its use as both a research and diagnostic tool has increased exponentially. PCR relies upon two oligonucleotide primers which are complementary to each strand of the double stranded DNA to be amplified. The distance between the two primers will determine the length of the product produced, which is termed an amplicon. The PCR amplification proceeds in the presence of dNTPs, *Taq* polymerase, magnesium ions, primers, template and the appropriate buffer conditions. There are three stages of the amplification reaction which require careful temperature control: denaturation of the double stranded DNA, annealing of the primer to the melted single strands, and extension of the newly synthesised product.

The initial denaturation temperature of around 94°C will denature the double stranded DNA leaving two separate strands. The second stage allows hybridisation of the primers to the template DNA and is typically 50-60°C. The third stage is an extension step of around 72°C which allows the *Taq* polymerase to extend from the 3' end of the annealed primer, generating a new complementary strand of DNA. This process is repeated several times, quickly generating many copies of the original template. As this process of rapid heating and cooling can be efficiently performed automatically in a thermocycler, the benefits of it were quickly realised (113, 185, 209).

PCR is now an established method in most branches of biology. It is a very rapid and sensitive method capable of amplifying fragments of DNA many times. With the purification of the thermal stable *Taq* polymerase, PCR became readily accessible to most laboratories. It is now used in a many clinical diagnostic laboratories for the detection of various pathogens, both bacterial and viral (31, 52, 121). It is also used in forensic analysis (19), in veterinary laboratories (83) and in molecular archaeological investigations. An example of this it the recovery of DNA from frozen samples from the 1918 influenza pandemic (228). Little was known about this particular virus until recently when well preserved corpses of people known to have died from this deadly virus were investigated (228). The samples obtained were

degraded, but amplification using PCR provided the first insight to this virus, showing it to be influenza A H1N1 (201).

Problems can occur in PCR however, with falsely negative or falsely positive results sometimes occurring (11). False negative results may arise when inhibitors to PCR are present in the sample. Some samples are more likely to be inhibitory, for example blood samples, as haemoglobin is a potent inhibitor of PCR. The extraction method used for obtaining the nucleic acid from the sample can greatly reduce the chance of getting a false result (98). There are various extraction methods, many of which are commercially available, and particular methods are more suited to certain sample types. For example, Fischer *et al* 1991 observed that silica columns provided a sensitive method which allowed direct comparison of viral loads between cellular and liquid specimens (77). These methods can remove most inhibitors from the sample allowing amplification to proceed.

Certain precautions can be taken against the appearance of falsely positive or falsely negative results. The laboratory layout for the extraction and amplification can be such that any chance of cross contamination from post-PCR products to fresh samples is negligible. If at all possible it is best for the procedures of extraction, amplification and post-PCR analysis to be conducted in separate rooms which have dedicated clothing and equipment. Also the design of the PCR can include anti-contamination measures such as the uracil-N-glycosylase (UNG) method which is often incorporated in commercially available PCR tests (59). The monitoring of each batch of samples with correctly placed negative and positive controls can provide an early indication of any contamination between samples. The use of internal controls can also guard against false negative results (discussed later in this chapter).

There are a variety of detection methods for post-PCR analysis. Most commonly gel electrophoresis is used, with visualisation of the amplicons achieved by ethidium bromide or sybrgreen staining and UV illumination. This method allows non-specific mis-priming in the PCR to be recognised by the appearance of unexpected bands on the gel. This is an extremely important consideration when designing and optimising a multiplex PCR reaction as extra

bands may confuse the interpretation of the result. Other detection methods are discussed later in this chapter.

Multiplex PCR

Multiplex PCR is the addition of several primer pairs to an amplification reaction with the aim of producing specific amplicons from each of the targets present in the starting sample. This technique was first described in 1988 (38). The majority of initial studies which used this approach were based on bacterial DNA genomes (149, 159) or were aimed at detecting gene mutations in inherited diseases (8, 38). More recently multiplex PCR has been used in the detection of viral pathogens (87, 124). One advantage of this technique is in saving time, as effectively many PCRs can be completed in the time it would otherwise take to perform a single amplification. The use of multiplex PCR also means that reagents and equipment are used more economically, and results can be obtained more readily and rapidly.

Another benefit of a multiplex PCR assay is that one or more nucleic acid targets can be detected in the same sample at the same time. Dual infections can, therefore, be diagnosed more rapidly. There are, however, certain difficulties inherent in the design and implementation of this method. It is not always straightforward to find primers which are efficient, specific, sensitive and do not interfere with one another. Therefore, the starting point of the project described in this thesis was to investigate several different primer pairs, and co-amplification conditions, in order to be able to define an efficient and usable multiplex PCR suitable for use as a diagnostic and epidemiology tool, to detect influenza and RSV.

Primer design

The first consideration of multiplex PCR primer design is that the primers must be as similar to each other in their physical properties as possible. These properties include the annealing temperature (Ta), GC content and length of the primers. The Ta is the temperature at which the primer dissociates from the DNA template and indicates the optimal annealing temperature range for the PCR reaction. Many primer design programs will calculate the optimal Ta which results in maximal primer binding without non-specific hybridisation or

primer dimerization. The melting temperature (Tm) of the product is also important. However all these properties are interdependent. For instance, the GC content of the primer will affect the Ta of the primer (61). The longer the primer, the higher the GC content and the more specific it is likely to be. A balance must therefore be struck between these two properties (61). Primers between 18 and 24 bases long tend to be suitable for most PCR assays, while allowing specific annealing and melting of the primer (61).

If the individual primer pairs have similar Ta and GC contents it is unlikely that they will require drastically different buffer and cycling conditions for optimal performance. There are several primer design packages including OLIGO and PrimerSelect (Lasergene) available which can help to calculate these variables. The size of the amplicon produced must also be considered when designing primers for a multiplex PCR. This parameter is especially important when more than one amplicon is to be produced and detected by agarose gel electrophoresis. Amplicons within a similar size range will be synthesised efficiently at similar rates, so it is best to design amplicons within a 1000 bp range (213). PCR products must be separated sufficiently to allow the products to be easily differentiated. The type of gel used will depend upon the size range of amplicons to be detected. Several different types of agarose gel can be used to separate fragments greater than 50 bp apart in a range of 100-1,500 bp. Polyacrylamide gels generally provide better results for fragments that are only a few base pairs apart in size (212).

The region of the genome amplified by the primers must also be chosen with care. The precise purpose of the PCR amplification will dictate the best target region to choose. Single stranded RNA viruses have genomes that mutate rapidly (discussed in Chapter 1), and so a conserved region for a diagnostic PCR assay is usually most appropriate. The increasing availability of sequence data from multiple isolates of the same virus helps the design of primers specific to these conserved regions. Software packages allow sequence databases to be searched for the possibility of mis-priming on other templates which may be present in the samples to be tested. Mis-priming on extraneous targets could lead to falsely positive results, or hard to interpret gels with multiple bands. In practice, testing of the primers with other

templates likely to be present in the sample set should be undertaken during the optimisation of a PCR assay.

Biochemical optimisation of PCR

Once the theoretical design of the primers and database searching has been done, testing and biochemical optimisation of them on suitable templates is necessary. Optimisation of amplification reactions is essential to determine specificity and sensitivity. In a clinical and forensic setting the samples available may only contain a small amount of target which may be of poor quality. Also, samples may have been in long term storage under non-optimal conditions, or been in transit a long time before arrival at the laboratory for testing. Therefore the assay must be as sensitive as possible to allow the best chance of detection of the target in the samples.

The constituent components of a PCR assay are the template, primers, DNA polymerase, buffer, magnesium ions and dNTPs. When setting up the PCR it is possible to control all of these constituents. In a clinical setting, however, little control over the amount of template added to the reaction is possible. DNA polymerases differ in their buffer requirements but a suitable buffer is usually supplied with the enzyme by the manufacturer. The concentration of magnesium ions and dNTPs are inter-related, as dNTPs bind magnesium ions. To function *Taq* polymerase requires free magnesium as well as the magnesium ions which are bound to dNTP and the DNA (111). Therefore the amount of dNTP in the reaction will effect its efficiency and so needs to be carefully optimised. Increasing the magnesium concentration can often show beneficial effects such as increased specificity and production of product, but may also have detrimental effects if the concentration is too high (111).

The optimisation of a PCR is best accomplished in a step wise fashion, and there are trouble shooting guides available to help this process (104, 111, 159). There are also commercial PCR optimisation kits available which aid this process e.g. the Stratagene Optiprime kit or the Sigma Optimisation kit. These kits provide a wide range of buffers, cations and other additives which may improve the PCR. The most widely used buffer system is TRIS with potassium ions, but there are alternatives such as ammonium (e.g. Q-buffer from Qiagen).

Altering the concentration of the *Taq* polymerase or the primers may also affect the PCR assay. Testing the primers over a wide range of annealing temperatures can provide a more specific reaction. A thermocycler which can provide a range of annealing temperatures through a gradient heating block facilitates this optimisation process (e.g. the Robocycler from Stratagene).

For many studies *Taq* polymerase induced errors in amplification reactions do not cause undue problems. The presence of a visible product is verification of the presence of the target in the sample and therefore sequencing of this target is not necessary. In studies where sequencing of the PCR product is necessary, reducing the amount of errors due to *Taq* polymerase is important. In sequencing studies one may assume that the error rate induced is uniform among the sequences analysed and therefore will not affect the comparison of these sequences. However when mutational studies are conducted, especially those where one base pair differences are important, the error rate of *Taq* polymerase becomes critical.

Hot start

The appearance of non-specific or unpredicted reaction products on a gel usually indicates that the DNA polymerase started synthesising DNA from other sources than the target. To prevent this occurring the technique of hot start was described by Nuovo *et al* (1991)(182). In a hot start one of the reaction components, usually the *Taq* polymerase, is withheld from the reaction mix until all of the components have reached the denauration temperature. Using this method a great increase in amplification specificity is usually observed (182).

The use of hot start in a PCR assay may provide more specificity to it as it reduces the chance of non-specific mis-priming occurring at the lower temperatures, which could lead to non-specific products being synthesised. There are several means by which a hot start can be achieved, and these can be divided into two categories: mechanical and non-mechanical.

Mechanical hot-start

The mechanical technique involves physically adding the missing reaction component when the reaction mix has reached the denaturing temperature. This technique has drawbacks as it involves an extra step which can be time consuming if there are large numbers of specimens. Contamination may also be a problem as the missing reagent needs to be added to the tubes whilst they are still in the thermocycler. This is a very important consideration, especially in nested reactions, which are more prone to carry over contamination than single round reactions.

Non-mechanical hot-start

Non-mechanical techniques involve the *Taq* polymerase enzyme being inactivated until the reaction reaches the denaturation temperature. This can be achieved by the binding of an anti-*Taq* polymerase antibody to the enzyme, which will only disassociate from the enzyme when the reaction reaches a high temperature (194). This has shown to be effective at preventing false negative results occurring with specimens with low target copy number (194). Chemical modification of *Taq* polymerase is another way of inactivating the enzyme until it reaches a certain temperature (e.g. *Taq* Gold, Perkin Elmer or Platinum *Taq,* Gibco). The advantage of these types of method is that no extra reaction vessel opening steps are involved (with the exception of mixing the antibody and *Taq* polymerase). However, with *Taq* Gold an activation step is required of between 10 and 30 minutes at 94°C, and these chemically altered polymerases are significantly more expensive than the ordinary polymerases.

Primer competition

It is extremely important that the primers do not mis-prime or compete with each other in any way. Mis-priming can lead to false positive results. Interference by one primer with another may lead to a loss in sensitivity of the reaction, and may also result in the synthesis of extraneous amplicons, which will be visible as bands if agarose gel electrophoresis is used as a method of detection. Interactions between primers may thus result in the generation of false negative or false positive results. It is therefore appropriate to test each primer pair on the individual templates before pooling them together in a multiplex reaction. This allows the

sensitivity of each primer pair to be determined, and also if any sensitivity is lost by the addition of the other primer pairs.

Internal controls

The addition of an internal standard to an assay allows quality control of it (177). Internal positive controls ensure that all reagents used in the assay were functioning properly. In contrast negative control reactions allow monitoring of carry-over contamination within the run. Positive controls will ensure that the overall quality of the run falls within specified standards. However positive controls do not allow the monitoring of the performance of the individual samples within the run. Therefore the use of internal controls ensures that each individual sample is correctly processed throughout the entire amplification procedure.

Internal controls are of two types. Firstly, there are those that are added to the sample upon receipt and prior to nucleic acid extraction (177). Secondly, there are those that are based on another target which should be present in the sample, besides the test target sequence (227). Internal controls of the first type have certain advantages. For example, the exact amount of the control added to the sample is known and can therefore be set towards the limits of detection for the assay. This will ensure maximum sensitivity is obtained throughout the entire procedure. With this type of control a negative result can only be due to an assay failure, and can therefore be investigated further. The sequence and size of this type of internal control should be as close to the target as possible to ensure that no difference in DNA structure or content accounts for any failure of amplification of the target. The internal control amplicon may be designed so that the same primer pair will amplify both it and the target amplicon. The amplicons are then distinguished by size or sequence. A drawback to this technique of adding an internal control to the sample is that it will not show if the sample was correctly taken. In the instance of nose and throat swabs a certain amount of cellular DNA should be present, however if the swab was incorrectly taken this will be absent, which increases the probability of the agent in question also being missing from the sample. A control added to the sample will only show that the assay is performing to set standards and will not reveal if the sample was adequately taken.

An internal control based on a housekeeping gene or target which should be present in abundance in the sample is the second option for the design of an internal control. The advantage here is that this target will have been treated in exactly the same way as the test target from collection to arrival at the laboratory for testing. It will give an indication that the sample has been taken correctly. Primers for several housekeeping genes are commercially available and facilitate the inclusion of this type of internal control in PCR assay. For example, β-actin primers are available from Stratagene. However, these primers need to work efficiently in the presence of the test target primers. A drawback of housekeeping genes as controls is that they could be absent in the presence of the target sequence. This may make interpretation of results which lack the control amplicon, but contain the target amplicon, difficult. Also, the sensitivity of detection in individual samples cannot be ascertained using this format of internal control, only that the procedure was adequate to amplify the control in that sample.

Amplicon detection methods

The method of detection of the amplicons produced from a PCR assay is also an important consideration when designing the primers. The most popular and easiest method for detection of the products is agarose gel electrophoresis and ethidium bromide staining of the DNA (124, 135). This method is cheap and does not require expensive specialised laboratory equipment; it is readily available to many laboratories. Electrophoresis differentiates the PCR products on the basis of molecular mobility, so the primers must be designed such that the resulting amplicons will be of sufficiently different size to allow easy separation and analysis. Other methods of detection involve the use of a probe that hybridises to the amplicon (72, 170). The probe must be designed so that it is sensitive and specific. Probes can be used in either the liquid phase (solution hybridisation) or in the solid phase, for example bound to the wells of a microtitire plate.

Solid phase detection

There are a few reports of the use of probes bound to a solid phase, but this method has mostly been applied to the detection of bacterial targets (162, 220). There are also some commercially available kits which offer diagnosis of the presence of pathogen nucleic acid

based on this method of detection of the amplicons. For example, the Amplicor HIV and HCV Amplicor tests from Roche. Solid phase detection has several advantages over gel electrophoresis/ethidium bromide staining. Firstly, it may be possible to use a 'universal' PCR to amplify several different types of target. This has been described for respiratory viruses (respiratory syncytial virus and parainfluenza 3 virus) where a single PCR amplification of the L gene produces an amplicon, which is distinguishable in the different virus types by specific probes in an ELISA format (72). This means that only one primer pair is used; the amplicons need not be of different size or be 'clean'. The individual targets are differentiated by the use of probes which are bound to a microtitre plate. This has also been described for *Camplyobacter* species, where PCR amplification of the 16S rRNA gene is followed by differentiation of the individual species using specific probes in an ELISA type format (170). Detection methods for the probes include colorimetric, chemiluminescent and fluorescent techniques. Secondly, solid phase detection provides greater sensitivity compared with the standard gel electrophoresis/ethidium bromide staining method (122, 162). With the use of multibiotinylated probes, sensitivity has been reported to be 100 fold more than that of gel electrophoresis (162). Also, the results can be quantified making analysis of them more objective, and quantitative assays can be constructed in a multiplex format as described by Fan *et al* (1998) where seven respiratory viruses were analysed in this format (75). With the addition of a probe, and therefore an extra selection step, the assay is more specific than gel electrophoresis.

DNA chips

A recent innovation for the detection of PCR products is the use of probes bound to a 'chip' (164). This is essentially a specialised glass slide onto which thousands of oligonucleotides are spotted or synthesised *in situ*. The PCR products are applied to the chip, allowed to hybridise to the probes, and detected by a fluorescent method. The position of the product bound to the probe on the slide is revealed by fluorescence and analysed by computer software. This technology is extremely useful for studies which involve screening very large numbers of samples (164). It is still in the developmental stage, and because of the current technology involved, very expensive; however, it has many attractive features. For example, if a large number of products can be detected on one chip, the saving in time in post-PCR

amplicon analysis is considerable, particularly as this is computer aided. The advantages of this method have already been highlighted by its use in the human genome project where it helps with analysis of the considerable amount of information produced (155). The benefits this technique can offer in patient management have recently been reported by Troesch *et al* 1999 where determination of both mycobacterial species and antibiotic resistance was conducted simultaneously (233).

Liquid phase detection

Probes in the liquid phase can be used in conjunction with thermocycler machines which can read fluorescent signals. Such apparatus includes the Lightcycler (LC), (261) and the ABI 7700 TaqMan (197). With this technique, either the accumulation of product binding a fluorescent dye (e.g. SYBR-green) or fluorescent resonant energy transfer (FRET) between reporter and quencher dye moieties bound to the probe is measured. FRET can be implemented in different ways, including bi-probes, hybridisation probes, hydrolysis probes and molecular beacons (235, 260).

The Lightcycler system can also be used for product accumulation and endpoint amplicon detection, and melting point analysis of the amplicon (203). The melting point analysis of amplicons produced can be measured and compared using Lightcycler software. This alone can provide a method for analysis of PCR products. It is theoretically possible to design a multiplex PCR capable of distinguishing amplicons based on their Tm. One of the advantages the Lightcycler has over any other amplification and detection method is that both amplification and detection can be performed at the same time in the same machine without any further practical intervention. Also, the time taken to perform a PCR and analysis is significantly faster than by any other method. The Lightcycler uses capillary tubes which only require small reaction volumes. A laser reads the light at a specified wavelength emitted from each capillary tube after every cycle. Therefore the accumulation of product can be measured in 'real-time'. This saving of time and reagents due to the small amounts used compared with conventional PCR techniques could be advantageous for large numbers of specimens. Additionally, the risk of contamination is reduced as the PCR and analysis is performed in a 'closed tube' environment. Therefore the amplicons produced with this

method should never be exposed to the external environment, reducing the contamination of equipment. A drawback is that the product is not readily recoverable from the capillary tube following amplification. Therefore, post amplification analysis of the product, such as sequencing, is not practical, particularly when large numbers of samples need to be analysed.

Aims

The work reported in this section of the thesis was to design primers capable of amplifying and subtyping RSV. These primers were then to be incorporated a multiplex PCR for influenza A (H1N1, H3N2) and influenza B (70). It was intended that when fully optimised the assay should be able to detect virus from low copy number and poor quality samples, such as nose and throat swabs. The amplicons from each individual sample should be clearly distinguishable by agarose gel electrophoresis. In addition the assay should be applicable to large numbers of clinical samples. Alternative detection methods to agarose gel electrophoresis were also explored; these included the use of Lightcycler and PCR ELOSA as a means of amplicon detection.

Results

Growth of RSV

RSV A (Long strain) was grown in Hep-2 cells, and distinctive CPE was observed. Several strains of RSV B were obtained from Dr. P Cane (University of Birmingham) for adaptation to growth in the laboratory. Three of four strains grew in Hep-2 cells, two Gambian strains showing very little CPE in cell culture and the one Birmingham strain showing typical RSV CPE. However, upon examination by either PCR or by infectivity assay, good growth of all strains was scored. The strains and properties of the viruses used in the development of the multiplex PCR are shown in Table 3.1.

Table 3.1 The growth properties of RSV and influenza strains used in the development of the multiplex PCR

Virus	Reference	Origin	Growth Conditions
RSV A	Long Strain	ECACC	Hep-2 cells
RSV B	VS-1039	Birmingham (UK)	Hep-2 Cells
Influenza A H1N1	A/TW/1/86	Taiwan (China)	Egg (allantoic)
Influenza A H3N2	A/Syd/5/97	Sydney (Australia)	Egg (allantoic)
Influenza B	B/Harbin/7/94	Harbin (China)	Egg (allantoic)

Optimisation of primer sets

The oligonucleotide primers designed to amplify RSV sequences were positioned in the N and P region of the genome (fig 3.1), as it is highly conserved and is one of the regions of the genome which allows subtyping of strains into A and B types (3, 126, 173). The GC content, Tm and length of the primers (Table 3.2) were chosen and analysed using the OLIGO 5 primer design program to ensure that they not only met the recommended criteria for optimal PCR primers (61) but also that they could be used in combination in a multiplex PCR reaction with conditions already determined to be effective for detection and subtyping of influenza A and B (70). Moreover, the primers were designed to ensure that the final reaction products could easily be differentiated on the basis of size from each other, and from the reaction products from amplification of influenza RNA templates.

Figure 3.1 Diagrammatic representation of the primer positions for influenza and RSV amplification

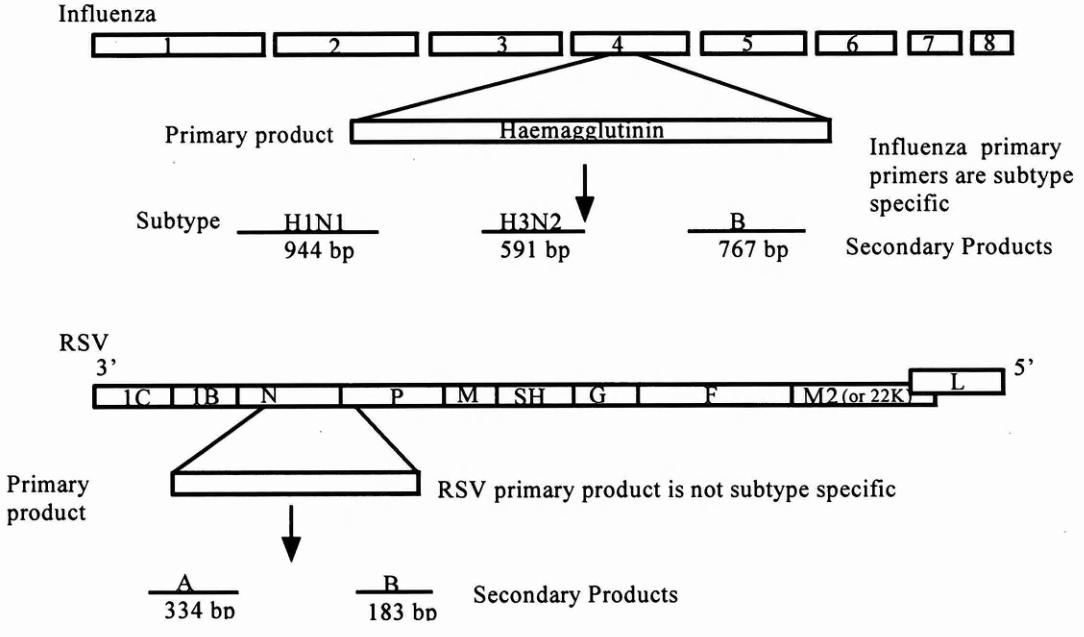


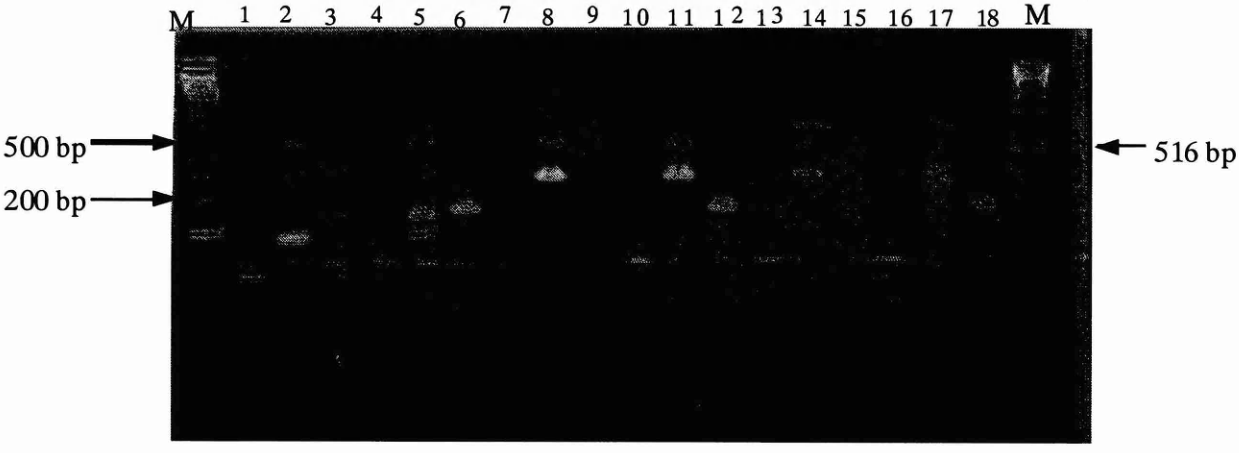
Table 3.2 Primers designed to amplify RSV N gene

Primer Name	Forward/ Reverse	Primary/ secondary	RSV Subtype	Gene Posistion	Nucleotide position*	%GC content	Ta °C	Sequence 5'→3'
RSV A/B 548	F	1°	A +B	548	1629	50	59.7	gtcttacagccgtgattagg
RSV A/B/1366	R	1°	A+B	1366	2447	45	60.9	gggctttctttggttacttc
RSVA610	F	2°	A	610	1691	50	60.7	ggcttactacccaaggacat
RSVA870	R	2°	A	870	1951	40	60	gggcatattcataaacctca
RSVB783	F	2°	B	783	1864	45	60.7	aatgctaagatggggagttc
RSVB1149	R	2°	B	1149	2230	45	65	tggggttgagttgatgcttt
RSV597F (B)	F	2°	B	597	1678	50	67.3	aaaacgctacaagggcctca
RSV984R(B)	R	2°	B	984	2065	60	67.8	ctgctgcattgcctaggacc
RSVA783	F	2°	A	783	1864	45	63.3	gatgttacggtggggagtct
RSVA1117	R	2°	A	1117	2198	35	46.2	gtacactgtagttaatcaca
RSVB966	R	2°	B	966	2047	35	54.3	gaaattgagttaatgacagc

Td calculated by using Oligo5 primer design package where Td is equal to Ta.
* On reference strain genbank accession number RSH1CE

Several primers were tested in various combinations (fig 3.2, Table 3.3) on laboratory grown material (Table 3.1). Some of the primers showed significant mis-priming on the RSV genome, as seen in fig 3.2 lane 5 which shows several unexpected bands when an RSV A template was amplified using primers A 783F, A 870R, B783F and B 966R, using the following PCR buffer conditions 10mM Tris-HCL (pH 8.4), 1.5 mM $MgCl_2$, 2.5 mM KCL and 0.75U *Taq* polymerase in both rounds of amplification with 0.2mM of each dNTP added to the second round amplification. Expected product sizes were: lane 2 - 87bp, lane 5 - 87 bp, lane 6 - 183bp, lane 8 - 340bp, lane 11 340 bp, lane 12 - 183 bp, lane 17 – 260 bp and lane 18 – 183 bp. All the rest should contain no amplification products.

Figure 3.2 Testing of potential primers designed to amplify RSV A and B



M is a marker with base pair sizes indicated with arrows. Primary amplification using primers RSV AB 548 &AB1366, secondary amplification using various combinations as follows Lane 2 RSV A amplified using primers A783 &A870. Lane 3 RSV B amplified using primers A783 & A870. Lane 5 RSV A amplified using primers A 783, A870, B783 & B966. Lane 6 RSV B amplified using primers A 783, A870, B783 & B966. Lane 8 RSV A amplified using primers A783 and A1117. Lane 9 RSV B amplified using primers A783 and A1117. Lane 11 RSV A amplified using primers A783, A1117, B783 & B 966. Lane 12 RSV B amplified using primers A783, A1117, B783 & B 966. Lane 14 RSV A amplified using primers A610 & B783. Lane 15 RSV B amplified using primers A610 & B783. Lane 17 RSV A amplified using primers A 610, A870, B783 & B966. Lane 18 RSV B amplified using primers A 610, A870, B783 & B966
Lane 1, 4, 7, 10, 13, 16 are negative controls.

Table 3.3 Final primer choice for RSV and influenza multiplex PCR

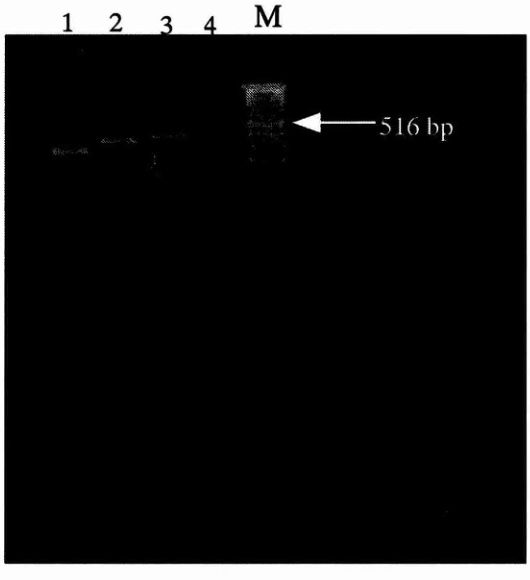
Primer	Sequence 5'→ 3'	Gene and nucleotide position (nt)*	Ta	GC content	Op. Ann. Temp. °C	Max. Ann. Temp. °C	Amplicon size	Sensitivity[a]
Primary amplification								
AHI A	cagatgcagacacaatatgt	HA gene (nt 76)	55	40	52	63	1015 bp	na
AHI FII	aaaccggcaatggctccaaa	HA gene (nt 1090)	72	50				
AH3 A	cagattgaagtgactaatgc	HA gene (nt 174)	55	40	52	62	883 bp	na
AH3 DII	gtttctctggtacattccgc	HA gene (nt 1056)	62	50				
BHA A	gtgactggtgtgataccact	HA gene (nt 154)	56	50	53	64	900 bp	na
BHA DII	tgttttcacccatattgggc	HA gene (nt 1053)	65	45				
RSV 548F	gtcttacagccgtgattagg	N gene (nt 1629)	63	50	52	66	838 bp	na
RSV 1366R	gggctttctttggttacttc	P gene (nt 2447)	64	45				
Secondary amplification								
AHI B	ataggctaccatgcgaacaa	HA gene (nt 96)	63	45	52	62	944 bp	<1
AHI EII	cttagtcctgtaaccatcct	HA gene (nt 1039)	55	45				
AH3 B	agcaaagctttcagcaactg	HA gene (nt 348)	63	45	54	69	591 bp	<1
AH3 CII	gcttccatttggagtgatgc	HA gene (nt 938)	65	50				
BHA B	cattttgcaaatctcaaagc	HA gene (nt 196)	61	35	54	67	767 bp	<1
BHA CII	tggaggcaatctgcttcacc	HA gene (nt 962)	68	55				
RSVA783F	gatgttacggtggggagtct	N gene (nt 1864)	64	45	48	51	334 bp	<1
RSVA1117R	gtacactgtagttaatcaca	N gene (nt 2198)	51	35				
RSVB783F	aatgctaagatggggagttc	N gene (nt 1864)	64	45	50	61	183 bp	<1
RSVB966R	gaaattgagttaatgacagc	N gene (nt 2047)	58	35				

a = Determined as PFU
Influenza muliplex primers were established byDr. Joanna Ellis (70)
* Nucleotide position for influenza based on genebank reference strain AF008656, nucleotide positions for RSV based on genebank reference strain RSH1CE

As a result of testing many different primer combinations, the primers shown in Tables 1 and 2 were selected for use in subsequent experiments. All the primers chosen were 20mers and had a GC content of less than or equal to 55% (Table 3.2 and 3.3). The primer sequences were also analysed using OLIGO 5 for the formation of dimers either within or between pairs, and none were predicted. Additionally, no significant theoretical mis-priming by any of the primers was identified with any database sequences.

Testing of the RSV A primers (RSV A 610F and RSV A 870 R) showed there to be competition with the RSV B primers when these pairs were combined in an amplification reaction with the RSV B template. This is shown as seen in lane 2 of fig 3.3 where the RSV B template amplified with primer pairs A 610, A 783, B 870 and B1149 shows a band of incorrect size (fig 3.3). the correct size band for RSV A is shown in lane 1 and the correct size band for RSV B is shown in lane 3. Several primary primer concentrations were tested in attempts to overcome this problem, but no improvement was seen.

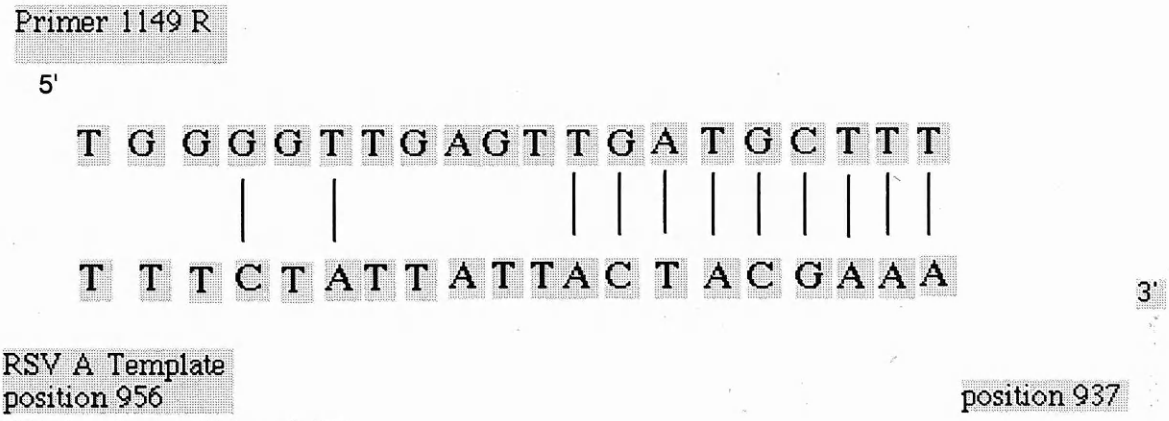
Figure 3.3 Competition between RSV primers in a multiplex reaction



Lane 1 RSV A amplified using primers 610 and 870. Lane 2 RSV B amplified using primers 610, 783, 870 and 1149. Lane 3 RSV B amplified using primers 783 and 1149. M is a marker with base pair sizes indicated with arrows.

Further multiplex PCR testing of the primers showed that the reverse RSV B primer (1149R) and the forward RSV A primer (610F) reacted to form a product from the RSV A template. Analysis using OLIGO 5 showed sequence similarity between the RSV B 1149 reverse primer and the RSV A template at nucleotide positions 937-956 (fig 3.4).

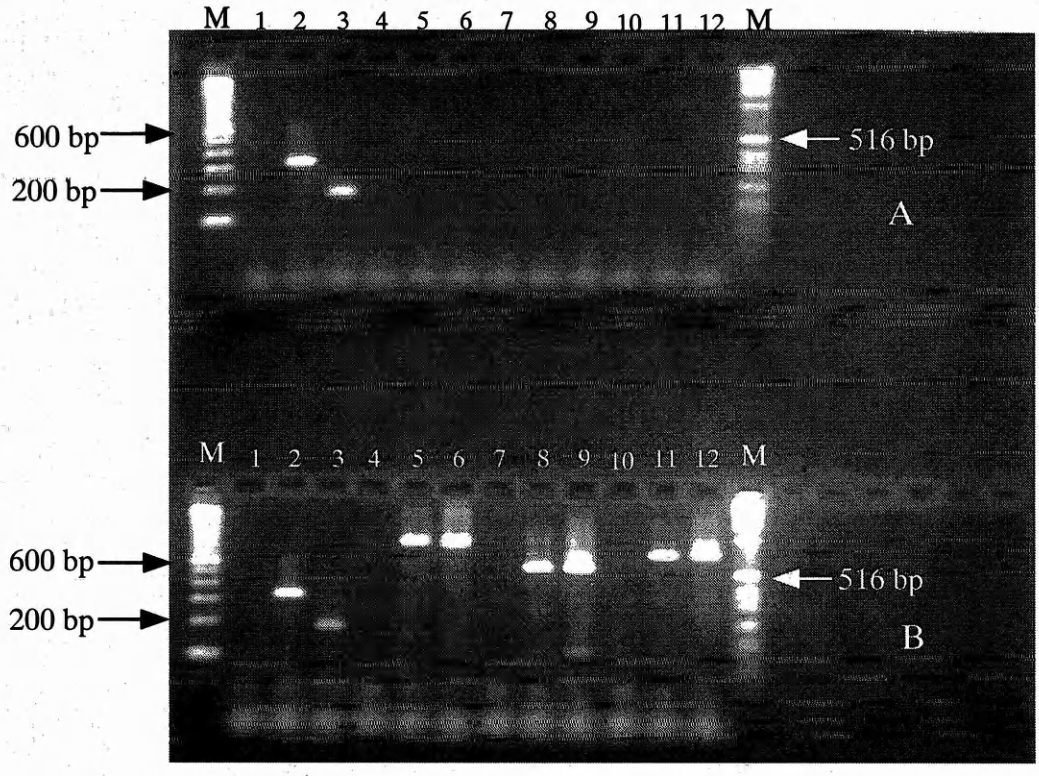
Figure 3.4 Diagrammatic representation of the mis-priming on the RSV B template due to



RSV A primers 610 and 870

Other primers were tested in many combinations on the RSV A template (Table 3.2). The RSV primers finally chosen are listed in Table 3.3. These primers were tested with influenza RNA templates (influenza A, H1N1, H3N2, and influenza B) and no mis-priming was observed (fig 3.5 A). Expected products of 340bp and 183 bp were seen in lanes 2 and 3 respectively. When all primers were tested in multiplex reactions, the intended templates were found to be correctly amplified, and each of the amplicon products was easily distinguishable on an agarose gel (fig 3.5 B). Expected products of 340bp (lane 2), 183 bp (lane 3), 944bp (lanes 5 and 6), 591 bp (lanes 8 and 9 and 767bp (lanes 11 and 12) were observed.

Figure 3.5 Final primer choice for RSV amplification



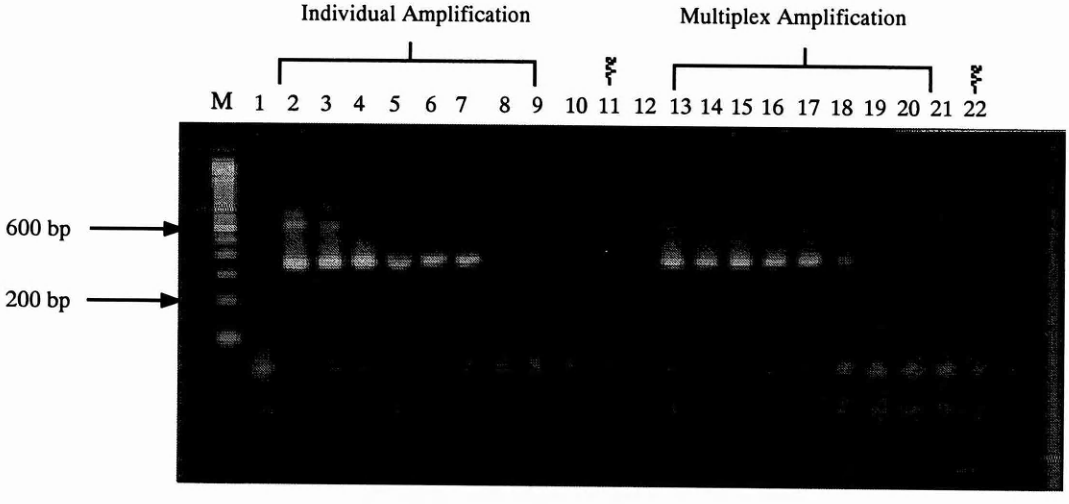
Panel A RSV primers only used to amplify the following templates: Lane 2 RSV A, Lane 3 RSV B, Lane 5 H1N1 (10^{-5}), Lane 6 H1N1 (10^{-2}), Lane 8 H3N2 (10^{-5}), Lane 9 H3N2 (10^{-2}), Lane 11 influenza B (10^{-5}), Lane 12 influenza B (10^{-2}).
Lanes 1, 4, 7, 10 are negative controls.
Panel B All multiplex primers used to amplify the following templates: Lane 2 RSV A, Lane 3 RSV B, Lane 5 H1N1 (10^{-5}), Lane 6 H1N1 (10^{-2}), Lane 8 H3N2 (10^{-5}), Lane 9 H3N2 (10^{-2}), Lane 11 influenza B (10^{-5}), Lane 12 influenza B (10^{-2}).
M is a marker with base pair sizes indicated with arrows. Lanes 1, 4, 7, 10 are negative controls.

Optimisation of PCR conditions

The variables tested were buffer conditions, Taq polymerase concentration, $MgCl_2$ concentration, annealing temperature and amplification conditions. The multiplex PCR containing the primer pairs for each template (Table 3.2) was then tested with a dilution series of each virus template. No loss in sensitivity was seen, as compared with the use of each primer pair individually. For example, with the individual templates the RSV A endpoint amplification was found to be the same using only the RSV A primers, as when all

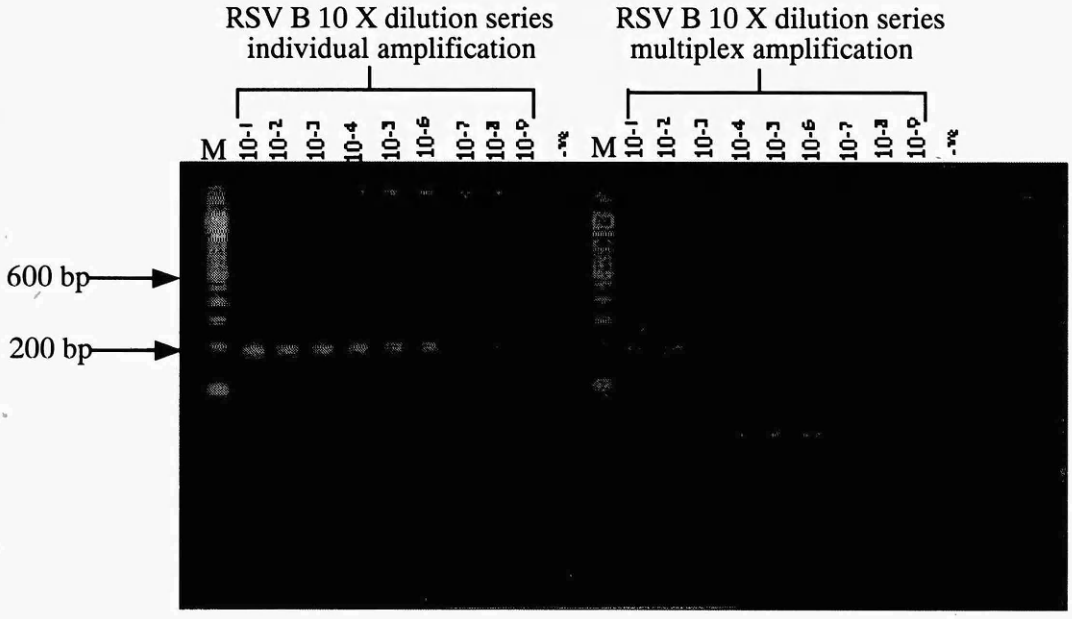
of the multiplex primers were present in the reaction (fig 3.6). The one exception to this was RSV B which showed a dramatic loss in sensitivity when in the multiplex reaction, in comparison to the single RSV B PCR (fig 3.7). The 10 fold dilutions were made from virus stocks shown in Table 3.1.

Figure 3.6 RSV A amplification by individual and multiplex PCR



Lanes 2-11 Serial 10 fold dilution series of RSV A tissue culture fluid (10^{-1} - 10^{-10}) amplified with just the RSV primers in a multiplex reaction. Lanes13-22 Serial 10 fold dilution series of RSV A tissue culture fluid (10^{-1} - 10^{-10}) amplified with all of the multiplex primers (table 2). No loss in sensitivity of detection is seen in the multiplex reaction with all of the primers. M is a marker with base pair sizes indicated with arrows. Lanes 1,12 and 23 contain negative controls.

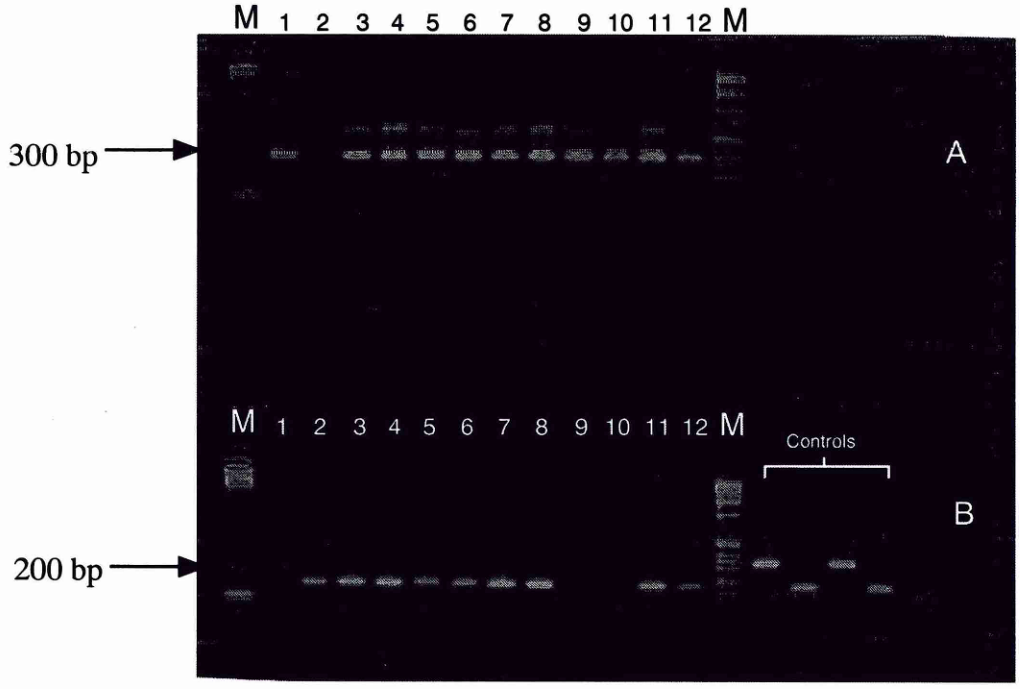
Figure 3.7 RSV B amplification by individual and multiplex PCR



Lanes 2-11 Serial 10 fold dilution series of RSV B tissue culture fluid (10^{-1}-10^{-10}) amplified with the RSV primers in a multiplex reaction. Lanes13-22 Serial 10 fold dilution series of RSV B tissue culture fluid (10^{-1}-10^{-10}) amplified with all of the multiplex primers (table 2). Loss in sensitivity of detection is seen in the multiplex reaction with all of the primers. M is a marker with base pair sizes indicated with arrows. Lanes 1, 12and 23 contain negative controls.

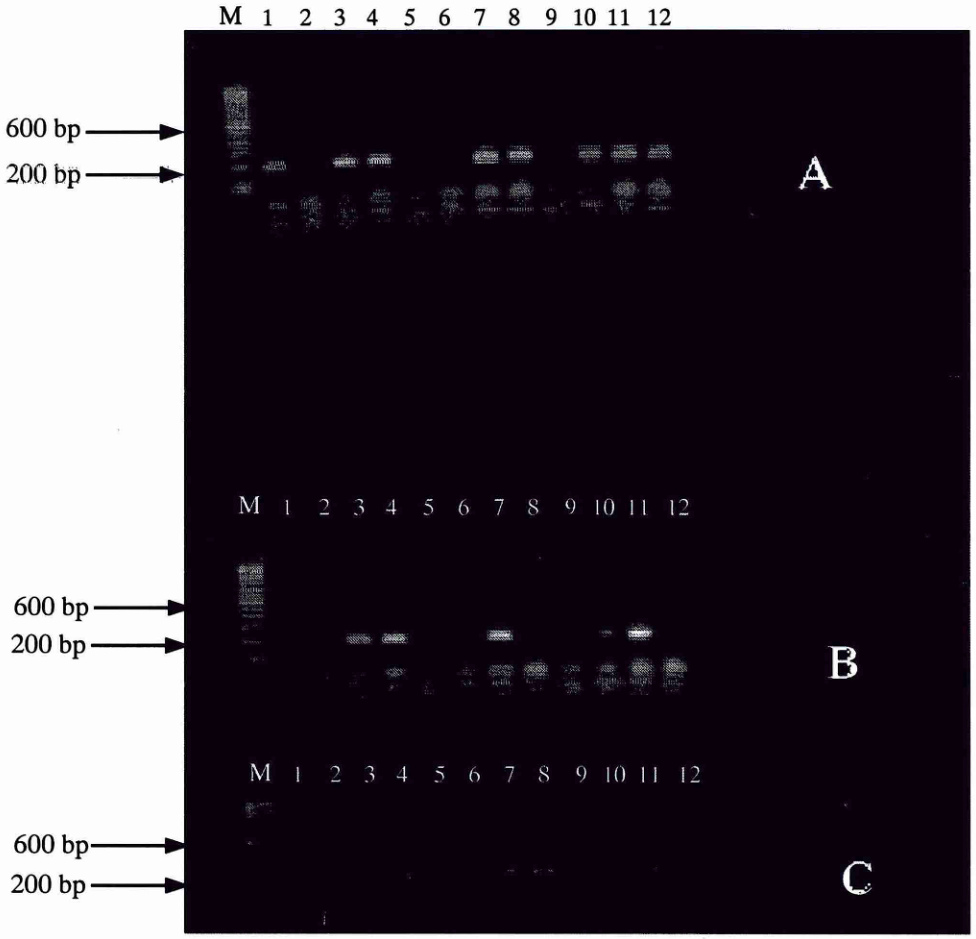
The RSV primers were assayed with a PCR optimisation kit (Stratagene) to ascertain the optimal buffer conditions for each primer pair with each individual template (fig 3.8). For example, in lane 2 panel A of figure 3.8, no amplification was seen when Optiprime buffer 2 was used. The end point of a dilution series of RSV A and RSV B RNA was determined using the same Optiprime panel to further define the optimal buffer (fig 3.9). As shown in figure 3.9, only 5 of the Optiprime buffers (lanes 3, 4, 7, 8, & 11) showed endpoint amplification. These buffers ranged in pH from 8.3 to 9.2 and all had 3.5 mM final magnesium ion concentration, in comparison with the other buffers which had 1.5 mM final magnesium ion concentration. Of the buffers which showed sensitive amplification, two were further tested as these gave the most intensely stained bands (lanes 7 and 11). These buffers both had final magnesium ion concentrations of 3.5 mM, 25 mM KCl and pH values of 8.8 (buffer 7) and 9.2 (buffer 11).

Figure 3.8 Optimisation of RSV primers using Stratagene Optimisation kit



Panel A Amplification of RSV A template (10^{-2} dilution of tissue culture fluid) using RSV A primers (A783 and A1117). Lanes 1 to 12 correspond with optiprime buffers 1 to 12. M is a marker with base pair sizes indicated with arrows.
Panel B Amplification of RSV B template (10^{-2} dilution of tissue culture fluid) using RSV B primers (B783 and B966). Lanes 1 to 12 correspond with optiprime buffers 1 to 12. M is a marker, arrows indicate marker sizes in bp.

Figure 3.9 End point sensitivity of the Stratagene Optiprime buffers

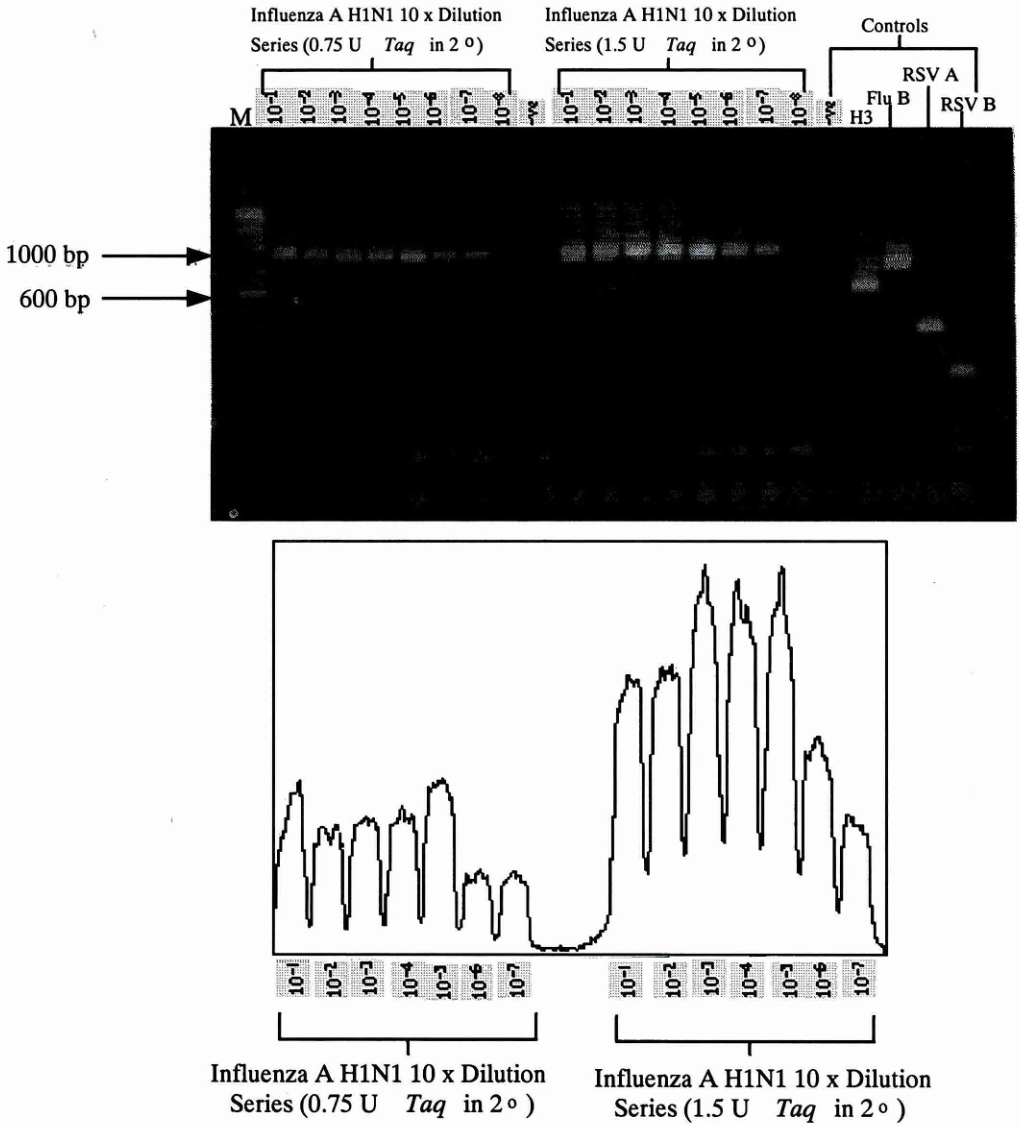


Panel A Amplification of RSV B (10^{-5} dilution of tissue culture fluid) using all multiplex primers (table 3.3). Lanes 1 to 12 correspond with optiprime buffers 1 to 12. M is a marker, arrows indicate marker sizes in bp.
Panel B Amplification of RSV B (10^{-6} dilution of tissue culture fluid) using all multiplex primers (table 3.3). Lanes 1 to 12 correspond with optiprime buffers 1 to 12. M is a marker, arrows indicate marker sizes in bp.
Panel C Amplification of RSV B (10^{-7}) using all multiplex primers (table 3.3). Lanes 1 to 12 correspond with optiprime buffers 1 to 12. M is a marker, arrows indicate marker sizes in bp.

Effect of increasing *Taq* polymerase concentration in amplification

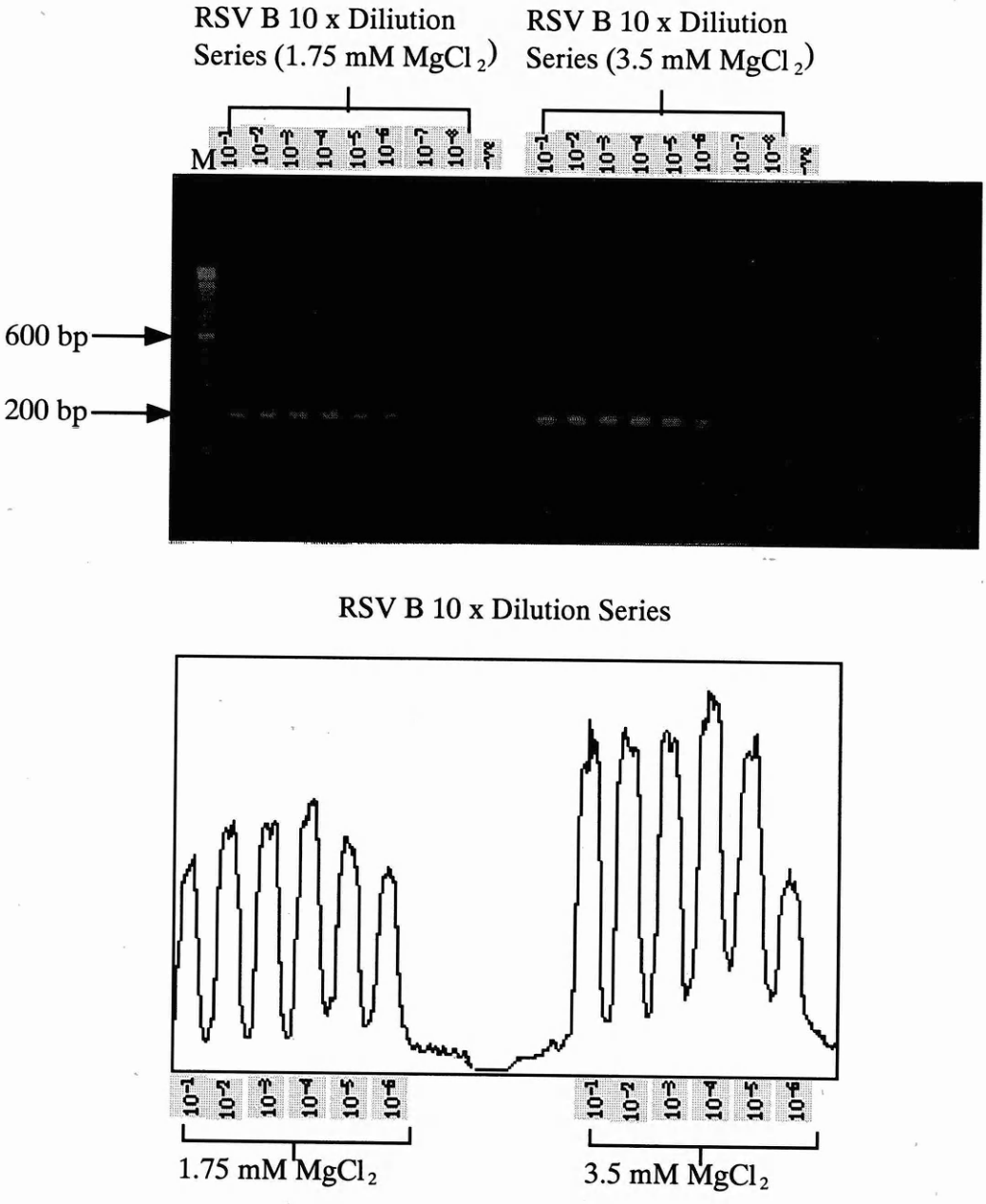
The effect of increasing the concentration of *Taq* polymerase in the secondary reaction was also determined and densitometry of the gel picture performed to measure this (fig 3.10). Although no increase in endpoint sensitivity was observed, the signal intensity was significantly increased with the increase in *Taq* polymerase. The effect of increasing the concentration of magnesium chloride in the secondary reaction was also determined (fig 3.11). No increase in endpoint sensitivity was observed, however the signal intensity was enhanced slightly with the higher magnesium ion concentrations. Increasing the amount of magnesium chloride and *Taq* polymerase in the same reaction showed the same results as increasing the amount of *Taq* polymerase alone. Influenza A H1N1 was used as it has the largest amplicon in the multiplex PCR.

Figure 3.10 Effect of *Taq* polymerase on amplification of influenza A H1N1



Serial 10 fold dilutions of influenza A H1N1 tissue culture fluid with each dilution amplified using all multiplex primers. 10 mM Tris-HCl pH8.4, 1.5 mM $MgCl_2$, 25 mM KCl and either 0.75U or 1.5 U *Taq* polymerase in the secondary reaction as indicated and 0.75 U *Taq* polymerase in the primary reaction Densitometry plot for each dilution amplified is shown M is a marker arrows indicate marker sizes in bp.

Figure 3.11 Effect of magnesium ion concentration on amplification on RSV B

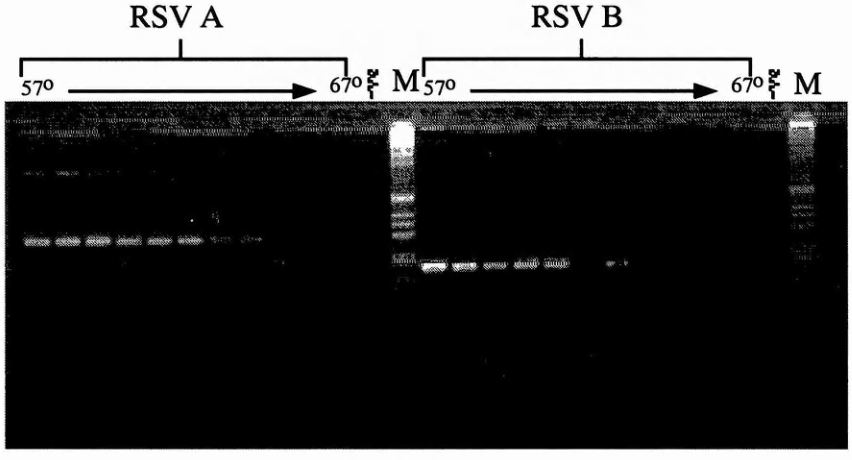


Serial 10 fold dilutions of RSV B tissue culture fluid with each dilution amplified using all multiplex primers with buffer conditions:10 mM Tris-HCl pH 8.8, 25 mM KCl, 1.5 U *Taq* polymerase in the primary and 0.75U *Taq* polymerase in the secondary reactions and either 1.75 mM $MgCl_2$ or 3.5 mM $MgCl_2$ as indicated. Densitometry plot for each dilution amplified is shown M is a marker arrows indicate marker sizes in bp.

Variation of primer concentration and buffer conditions

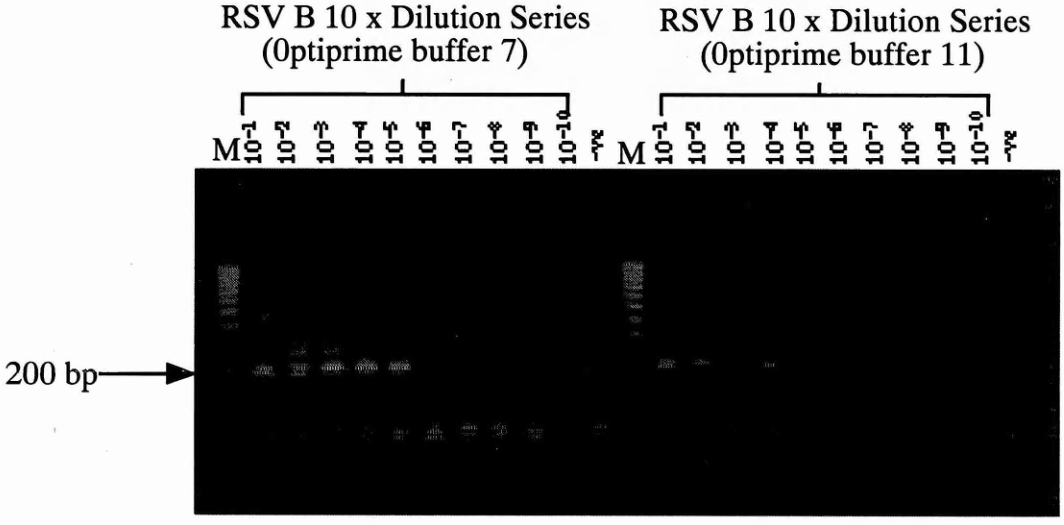
The concentration of the RSV B secondary primers was varied with no significant improvement being observed with higher concentrations of primer. In these experiments the primers in the first round amplification were used at 5 pmol per reaction. Qiagen ammonium buffer was also tested (Q-Solution) (138) but no gain in sensitivity was seen. A range of annealing temperatures were tested for both sets of RSV primers (fig 3.12). The finally chosen annealing temperatures were 50°C for the primary reaction and 60°C for the secondary reaction. Increasing the concentration of dNTP in the primary reaction showed no improvement in sensitivity. Both Optiprime buffer 7 and 11 were tested on dilution series of viruses, with Optiprime buffer 7 showing the best increase in sensitivity in the multiplex reaction with the RSV B template (fig 3.13). The sensitivity of detection when Optiprime buffer 7 was used with the other 4 viral templates (influenza A H1N1 and H3N2, influenza B and RSV B) was not changed.

Figure 3.12 Testing of optimal primer annealing temperatures of multiplex primers



Annealing temperature variation 57-68°C in the secondary reaction. Amplification performed using all multiplex primers on both RSV A and RSV B. M is a marker with base pair sizes indicated with arrows

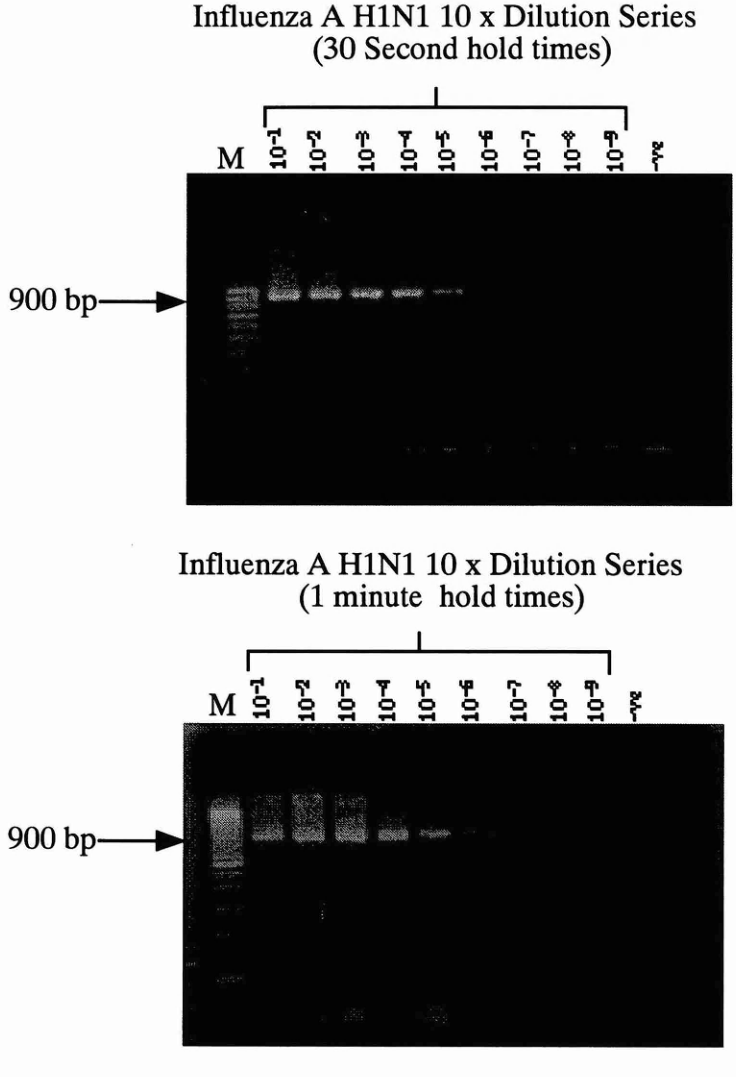
Figure 3.13 Sensitivity testing of Stratagene Optiprime buffers 7 and 11



Serial 10 fold dilution series of RSV B tissue culture fluid, each dilution is amplified with all multiplex primers using either buffer 7 (10 mM Tris-HCl pH 8.8, 3.5 mM $MgCl_2$, 25 mM KCl and 1.5 U *Taq* polymerase in both the primary and secondary reactions) or buffer 11 (10 mM Tris-HCl pH 9.2, 3.5 mM $MgCl_2$, 25 mM KCl and 1.5 U *Taq* polymerase in both the primary and secondary reactions). M is a marker with base pair sizes indicated with arrows

Decreasing the cycling times to 30 seconds and 45 seconds hold at each stage of the reaction was tested, and a loss in sensitivity was seen with the largest product (H1N1) using 30 second hold time as compared with using 1 minute hold time (fig 3.14). A summary of the optimisation steps investigated is given in Table 3.4.

Figure 3.14 Amplification of influenza A H1N1 using 30 second hold times



Serial 10 fold dilution series of influenza A H1N1 egg material, amplification was performed using all multiplex primers with 30 second hold times at each stage of amplification. M is a marker with base pair sizes indicated with arrows

Table 3.4 Optimisation Summary

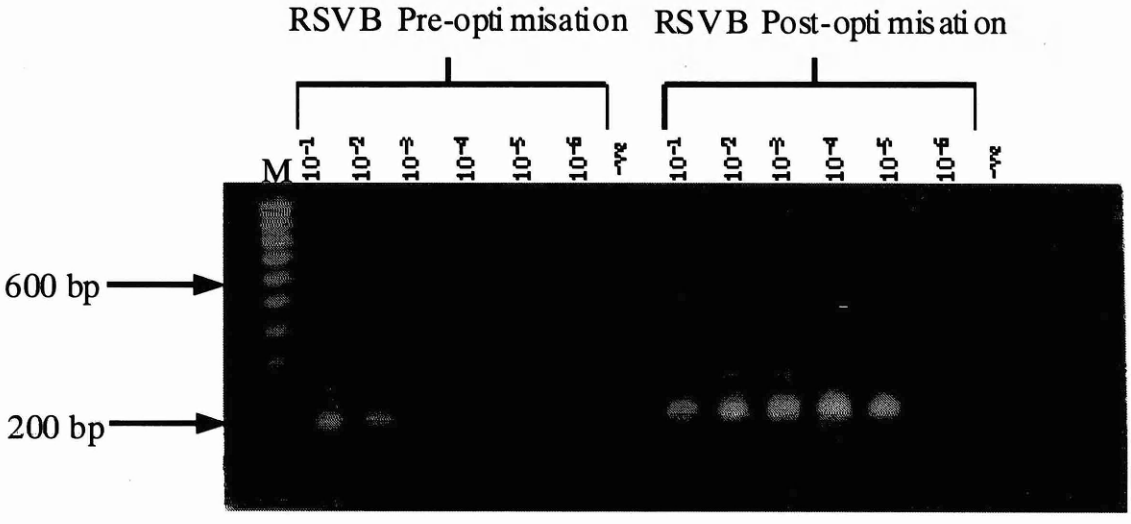
Condition Varied	Template					
	Influenza A H1N1	Influenza A H3N2	Influenza B	RSV A	RSV B	Figure/ Ref
.75U to 1.5U *Taq*	+	+	+	+	+	Fig 3.10
1.75 mM to 3 mM $MgCl_2$ Concentration	+	ND	ND	ND	+	Fig 3.11
1.5U *Taq* and 3mM $MgCl_2$ Concentration	+	ND	ND	+	+	NS
Gradient annealing Temperature	ND	ND	ND	+	+	Fig 3.12
Altered secondary primer concentration	ND	ND	ND	ND	+	NS
Optiprime panel & adjuncts	ND	ND	ND	+	+	Fig 3.8
Optiprime buffer 7 & increased *Taq*	+	+	+	+	+	Fig 3.13
Mis-priming testing	+	+	+	+	+	Fig 3.5
Cycling alterations	+	ND	+	+	+	Fig 3.14

ND = Not Done NS = Not shown + = Tested

Final multiplex RT-PCR reaction conditions

The pre-optimisation amplification conditions were compared with the post-optimisation conditions. A representative comparison of sensitivity of detection for RSV B is shown in figure 3.15 in which a three fold increase in sensitivity can be achieved. PCR in 10 mM Tris-HCl pH 8.8, 3.5 mM $MgCl_2$, and 2.5 mM KCl (Optiprime buffer 7) and using I.5U *Taq* polymerase (Gibco-BRL) was found to be optimal. The final amplification protocol included an initial denaturation at 94°C for 2 min, then had 35 cycles of 94°C for 1 min, 50°C for 1 min and 72°C for 1 min for the primary reaction; and an initial denaturation at 94°C for 2 min, then 35 cycles of 94°C for 1 min, 60°C for 1 min and 72°C for 1 min for the secondary reaction.

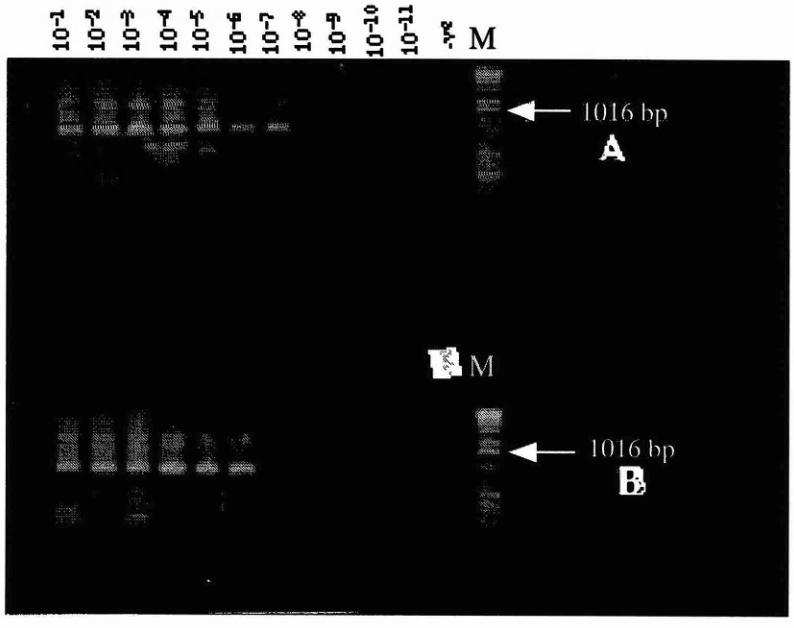
Figure 3.15 A comparison of pre and post optimisation amplification of RSV B templates



Serial 10 fold dilution series of RSV B tissue culture fluid each dilution is amplified with all multiplex primers. Pre-optimisation conditions:10 mM Tris-HCl pH8.4, 1.5 mM $MgCl_2$, 25 mM KCl and 1.5 U *Taq* polymerase in the primary reaction 0.75 U *Taq* polymerase in the secondary reaction. Post-optimisation conditions: 10 mM Tris-HCl pH 8.8, 3.5 mM $MgCl_2$, 25 mM KCl and 1.5 U *Taq* polymerase in both the primary and secondary reactions. M is a marker with base pair sizes indicated with arrows

Mechanical means of hot start for the multiplex RT-PCR were not attempted, as the assay was designed for use with large numbers of samples, which would make such approaches impractical. Non-mechanical means of hot start were tested, including Taq-start antibody (Clontech) and Ampli-Taq Gold (Perkin Elmer). No improvement in sensitivity or specificity (fig 3.16) was seen with either of these hot start methods and, therefore, no form of hot start was incorporated in the multiplex RT-PCR.

Figure 3.16 A comparison of hot start amplification with a non hot start amplification of influenza A H1N1

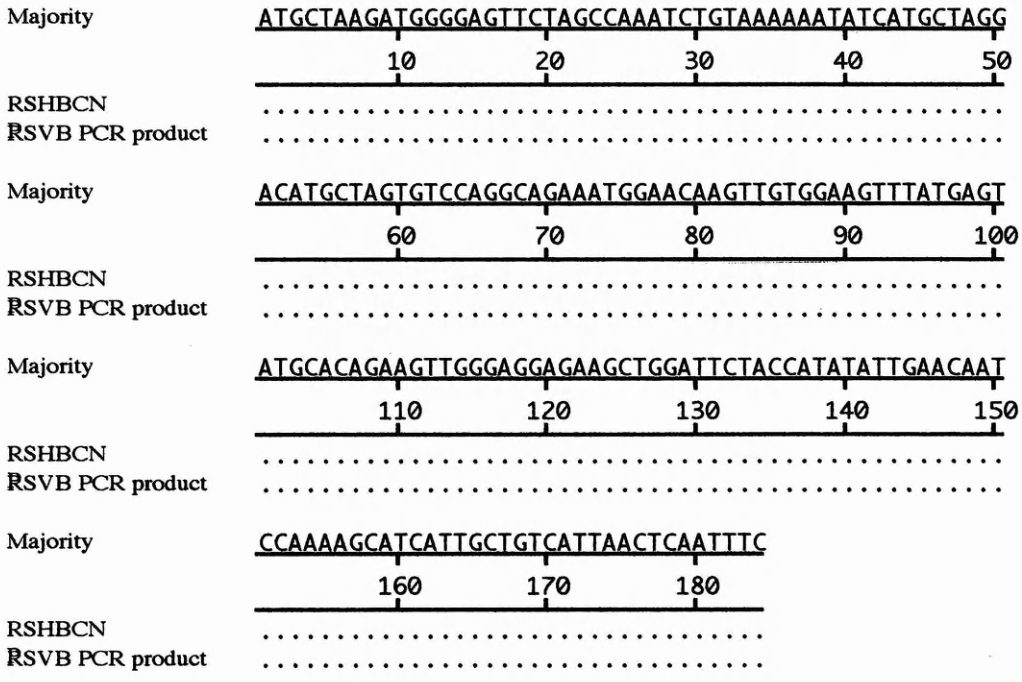


Panel A Serial 10 fold dilution series of influenza A H1N1 amplified without hot start
Panel B Serial 10 fold dilution series of influenza A H1N1 amplified with TaqStart antibody. M is a marker with base pair sizes indicated with arrows

Specificity and validation

The multiplex RT-PCR was tested for specificity with all of the viral RNA targets (influenza A H1N1, H3N2 and B, and RSV A and B) using first the RSV primers, and then adding each of the influenza primer pairs sequentially, with simulated clinical specimens. No mis-priming was observed when all of the primer sets were present with either influenza A or B or RSV A or B templates (fig 3.5). A product of the expected size was obtained with each viral template using the multiplex RT-PCR with all of the primer sets present (fig 3.5). The specific products could all be clearly separated and identified on a 2.25% NuSieve agarose gel (fig 3.5). This was found for both laboratory adapted virus control material (tissue culture grown virus, influenza and RSV, or egg grown influenza) and for clinical samples containing wild type strains. The product specificity of the amplicons obtained from multiplex RT-PCR reactions was also confirmed by sequence analysis with RSV B shown as an illustration (fig 3.17).

Figure 3.17 Alignment of the RSV B PCR product with sequence database strain RSHBCNP

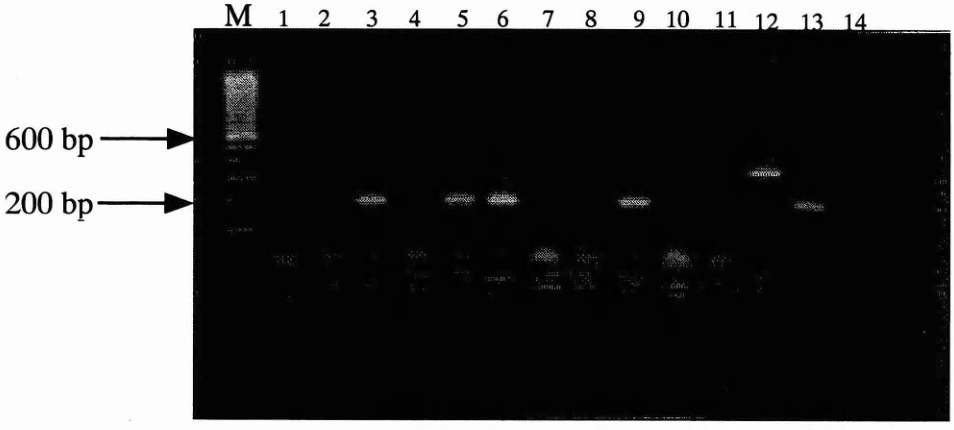


RSHBCN is a database strain accession no. D00736

No detectable PCR product was found after nucleic acid extraction and multiplex RT-PCR amplification on 40 clinical samples, (nasopharyngeal aspirates, nose and throat swabs or broncholalveolar lavage material) as specified on pg 40. Fifteen of these samples contained human parainfluenza viruses 1-3, seven had human CMV, four had HSV 1, three had untyped enteroviruses and 11 had rhinoviruses. The multiplex RT-PCR was tested blind on a panel of 65 nasopharyngeal aspirates (NPA), broncheoaveolar lavages and endotracheal aspirates. Forty of them contained RSV A and 20 contained RSV B as determined by ELISA using RSV type specific monoclonal antibodies.

An example of the results obtained is shown in figure 3.18. As controls there were five negative NPAs from which no virus was recovered. There was 100% correlation between the RSV subtype as determined by PCR and as determined by subtype specific ELISA. There was also 100% correlation between PCR type and antigenic type determined for 100 nose and throat swabs containing influenza A H1N1, H3N2 or influenza B virus. The antigenic typing of influenza virus grown in tissue culture from the original specimen was performed using haemagglutination inhibition (HI) with post-infection ferret antisera (36). Culture negative specimens were also studied and it was found that the multiplex PCR was more sensitive than culture methods for both influenza and RSV.

Figure 3.18 Testing of the multiplex PCR on clinical samples



Lane 1 and 2 are combined nose and throat swabs containing parainfluenza virus. Lanes 3,5,6 and 9 are broncheoaveolar lavages containing RSV B. Lanes 8 and 10 are nasopharyngeal aspirates containing 1 untyped enterovirus (Lane 8) and 1 rhinovirus (lane 10). Lanes 4, 7 and 14 are negative controls. M is a marker with base pair sizes indicated with arrows

Determination of sensitivity

The sensitivity of detection of influenza virus and RSV with nested primer sets used individually and in a multiplex reaction was determined. Ten fold dilution series of freshly harvested tissue culture fluid (RSV or influenza) or egg grown virus (influenza only) were prepared in VTM. Nucleic acid was immediately extracted for cDNA synthesis from 100-150 μl of each dilution. An equivalent volume of each dilution was taken for infectivity assays for both RSV or influenza, which were set up on the same day. Complementary DNA synthesis was followed by PCR with primer sets used individually and in multiplex reactions. Thus, the end point of detection of infectious virus could be directly compared with the end point of detection of viral RNA by multiplex RT-PCR. As only 50% of the cDNA obtained from each extraction was amplified in each PCR reaction, the PCR endpoint was described as a PCR D50 endpoint.

In practice, for both influenza and RSV, in the presence all five primer sets RT PCR detected viral nucleic acid at one to two ten-fold dilutions below the last dilution at which infectious virus particles could be identified. Therefore, the multiplex RT-PCR was capable of reliably detecting one or less pfu of influenza A (H1N1 or H3N2), influenza B, RSV A and B. In all cases, the end point of multiplex RT-PCR detection for the specific targets was unaltered by the presence of the other primer sets in a multiplex reaction.

Preparation of frozen PCR mixes

Preparation of individual reaction mixes containing five primer sets for the primary and secondary PCR reactions, for a large number of tests, was found to be very time consuming and prone to error. To overcome this problem and to facilitate the performance of multiplex RT-PCR testing in diagnostic settings, I investigated the preparation and storage of reagent 'master mixes'. Solutions containing all components of the primary or secondary reaction mixes, excluding *Taq* polymerase, were aliquoted in amounts sufficient to complete 15 PCR reactions, and were stored in sterile screw top Sarstedt tubes at -20 °C. The sensitivity of detection of the largest PCR product, derived from the influenza A H1N1 viral template (944bp) and low copy number controls of influenza A H3N2, influenza B and RSV A and B, was compared for PCR sensitivity with reagent mixes which had been stored at -20 °C for between one and six months, and for PCR sensitivity with freshly prepared reaction mixes. The frozen reaction mixes were defrosted either at room temperature or in a 37°C heating block prior to the addition of *Taq* polymerase, and the reaction completed as usual. No significant difference in the sensitivity of detection of the H1N1 reaction product could be seen qualitatively in the last dilution at which H1N1 could be detected (fig 3.19). Densitometry was performed but did not reveal any more information than the original gel image. Low copy number controls for influenza A H3N2 and influenza B and RSV A and B were also as reliably detected by PCR with the frozen reagent mixes as by PCR with the freshly made reagents.

Figure 3.19 Master mix amplification testing

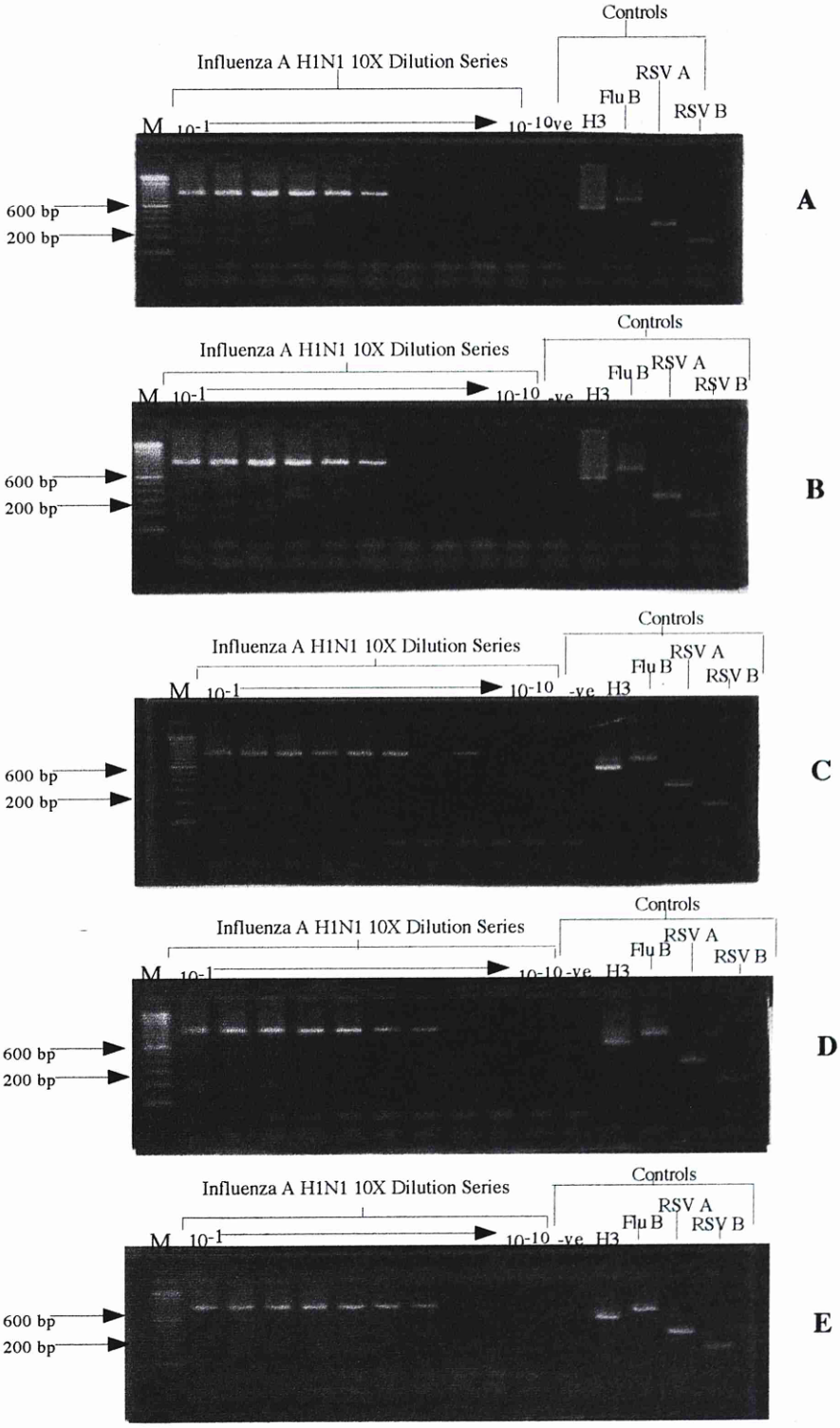


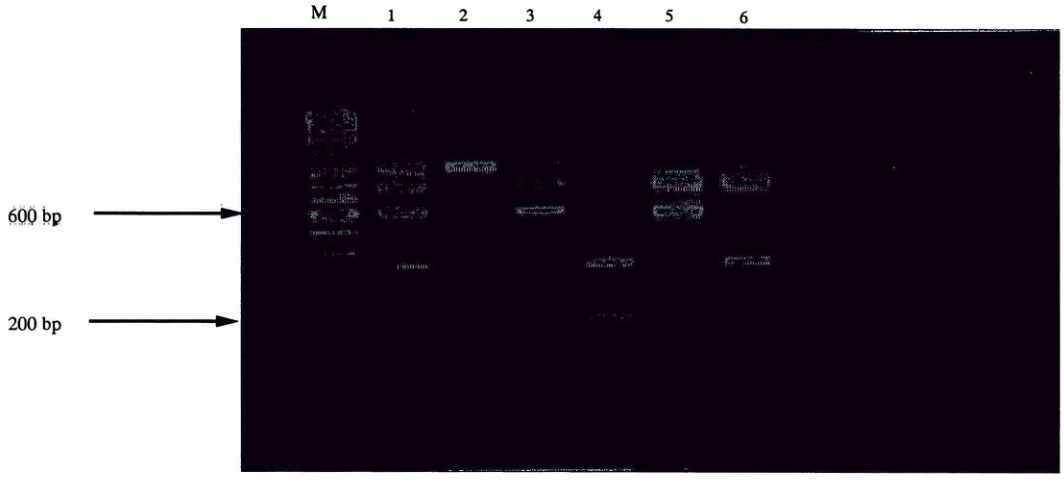
Figure 3.19
Panel A
A serial 10 fold dilution series of influenza A H1N1 amplified in a multiplex reaction using all primer sets in freshly prepared reaction mixes.
Panel B
A serial 10 fold dilution series of influenza A H1N1 amplified in a multiplex reaction using all primer sets in reaction mixes frozen at -20°C for 1 month.
Panel C
A serial 10 fold dilution series of influenza A H1N1 amplified in a multiplex reaction using all primer sets in reaction mixes frozen at -20°C for 2 months.
Panel D
A serial 10 fold dilution series of influenza A H1N1 amplified in a multiplex reaction using all primer sets in reaction mixes frozen at -20°C for 3 months
Panel E
A serial 10 fold dilution series of influenza A H1N1 amplified in a multiplex reaction using all primer sets in reaction mixes frozen at -20°C for 6 months.
M is a marker with base pair sizes indicated with arrows

Dual infections

The ability of the multiplex PCR to detect the presence of more than one viral template in the same starting material was assessed by the preparation of negative nose and throat swabs with known virus material placed into them in various combinations by Dr. M. Zambon. The multiplex reaction was capable of detecting all five templates simultaneously (fig 3.20), as well as various combinations of templates of simulated clinical material (fig 3.20). This indicated that co-infections could be detected with multiplex RT-PCR. Detection of all five targets required an increased concentration of *Taq* polymerase in the secondary reaction from 1.5 U to 3 U. Using 1.5 U *Taq* polymerase in the secondary reaction resulted in the non-specific loss of one template.

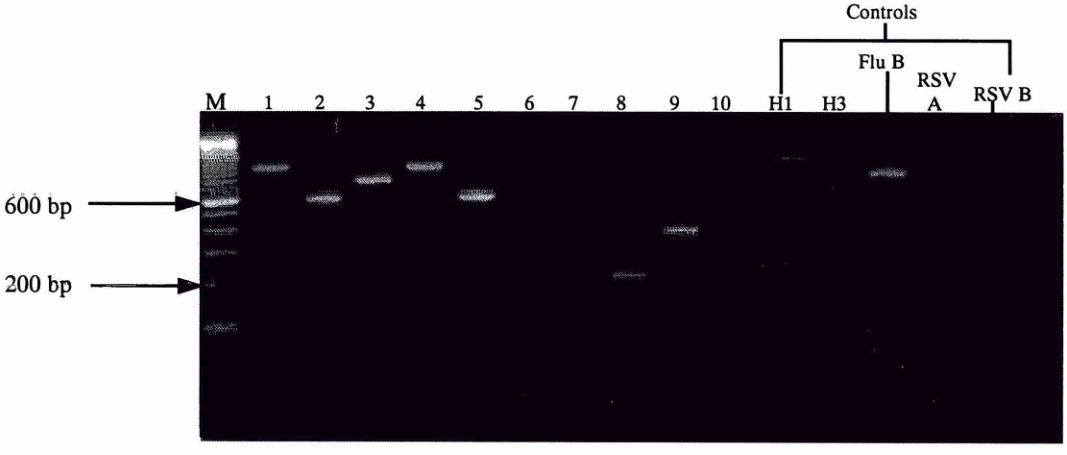
Testing of a prepared panel of clinical samples blind gave 100% correlation with the expected results (fig 3.21). For example, the specimen in lane five consisted of a negative combined nose and throat swab spiked with 1.5×10^{-3} pfu per ml of influenza A H3N2. Amplification of this sample in a multiplex reaction showed a product of the expected size at 591 bp.

Figure 3.20 Simulated dual infection amplification



Multiplex PCR (with all primer sets) was performed on combined nose and throat swabs spiked with various combinations of influenza and RSV. Lane 1 contains all 5 viral templates (influenza A H1N1, H3N2, influenza B and RSV A and B), Lane 2 contains influenza A H1N1 and RSV B, Lane 3 contains Influenza A H1N1, H3N2 and influenza B, Lane 4 contains RSV A and B, Lane 5 contains RSV A and B, Lane 5 contains Influenza B and influenza A H3N2, Lane 6 contains influenza B and RSV A. M is a marker with base pair sizes indicated with arrows

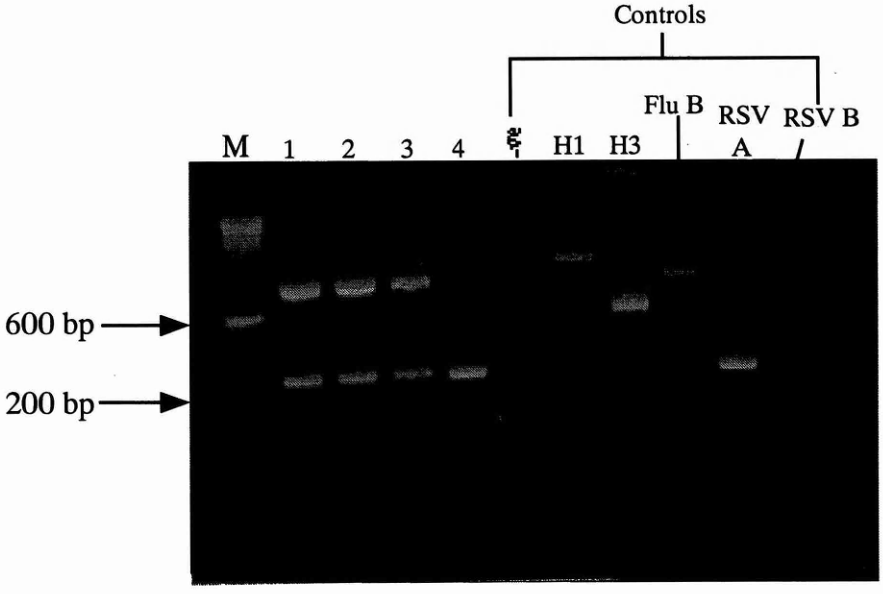
Figure 3.21 A blind panel of spiked samples amplified with the multiplex PCR



Multiplex PCR (with all primer sets) was performed on combined nose and throat swabs spiked with various combinations of influenza and RSV. Lane 1 contains influenza A H1N1, lane 2 contains influenza A H3N2, lane 3 contains influenza B, lane 4 contains influenza A H1N1, lane 5 contains influenza A H3N2, lane 6 contains RSV B, lane 8 contains RSV B and lane 9 contains RSV A. Lanes 7 and 10 are negative controls. M is a marker with base pair sizes indicated with arrows

Sequential specimens from an immunocrompromised child displaying respiratory illness were tested by multiplex PCR, culture and immnuofluorescences (DIF) (fig 3.22). The child was shown to have detectable RSV and influenza in all four samples by multiplex PCR. The corresponding culture and DIF results gave conflicting results for all but the last sample.

Figure 3.22 Sequential specimens from an immunocompromised child amplified with the multiplex PCR; the corresponding culture and IF results are shown.



Culture	RSV	-	-	-	+
	Influenza B	+	+	+	-
DIF	RSV	+	+	+	+
	Influenza B	-	-	-	-

Lanes 1 to 4 sequential samples from an immunocompromised child, corresponding culture and DIF results are shown. Sample types were bronchoalveolar lavage (lane 1), endotracheal aspirate (lane 2), and nasopharyngeal aspirate (lanes 3 and 4). M is a marker with base pair sizes indicated with arrows

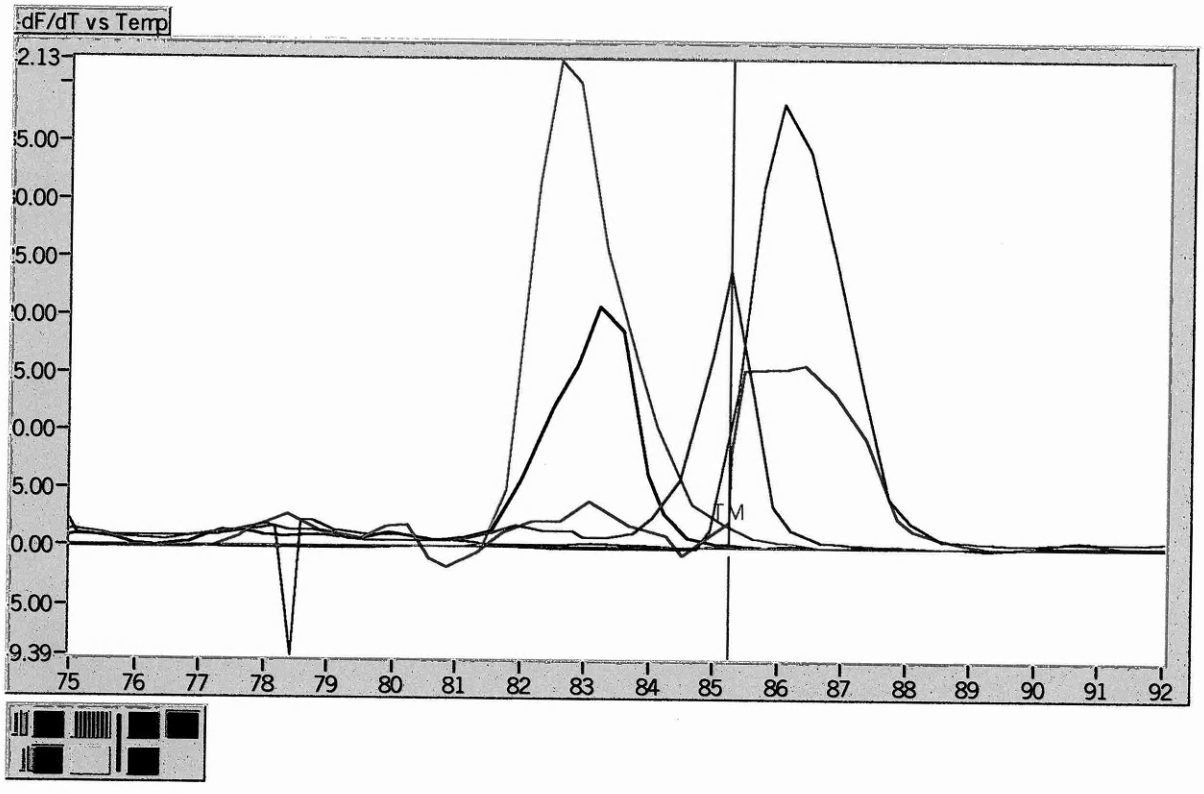
Alternative detection methods

Lightcycler

The use of real time PCR in the Lightcycler was investigated to determine if this would simplify and streamline the multiplex PCR. Standard amplification times in the Lightcycler consisted of 30 cycles of 95°C no hold, 60°C for 2 seconds and 72°C for 10 seconds. The buffers used were supplied with the *Taq* polymerase by BioGene. Sybrgreen was used at a 1 in 10,000 dilution to measure the accumulation of product. The reaction components for a 10 µl reaction were 5 µl buffer, 0.5 µl sybrgreen (1/10,000) 1µl each primer (5 uM) and 2.5 µl template.

PCR from cDNA from each template amplified with either the individual outer primer pairs or the multiple primer pairs proved unsuccessful even when 70 cycles were used. Therefore the primary reaction product was added to secondary mixes with a range of $MgCl_2$ concentrations (3-10mM). Primary amplification was performed on the conventional block thermocycler. To reduce the starting fluorescence a low dilution of the primary product was used (10^{-7}). No amplification could be seen with any of the primers. Therefore each primer set was tested individually to assess the melting point of each amplicon (fig 3.23, Table 3.5). All of the $MgCl_2$ concentrations showed amplification except 1 mM, 9 mM, and 10 mM. For individual amplification reactions 3 mM $MgCl_2$ was used and in the multiplex amplification reaction 5 mM $MgCl_2$ was used. Amplification was checked via agarose gel electrophoresis of the amplicons.

Figure 3.23 Melting point analysis of RSV and influenza amplicons performed in an individual reaction on the Lightcycler



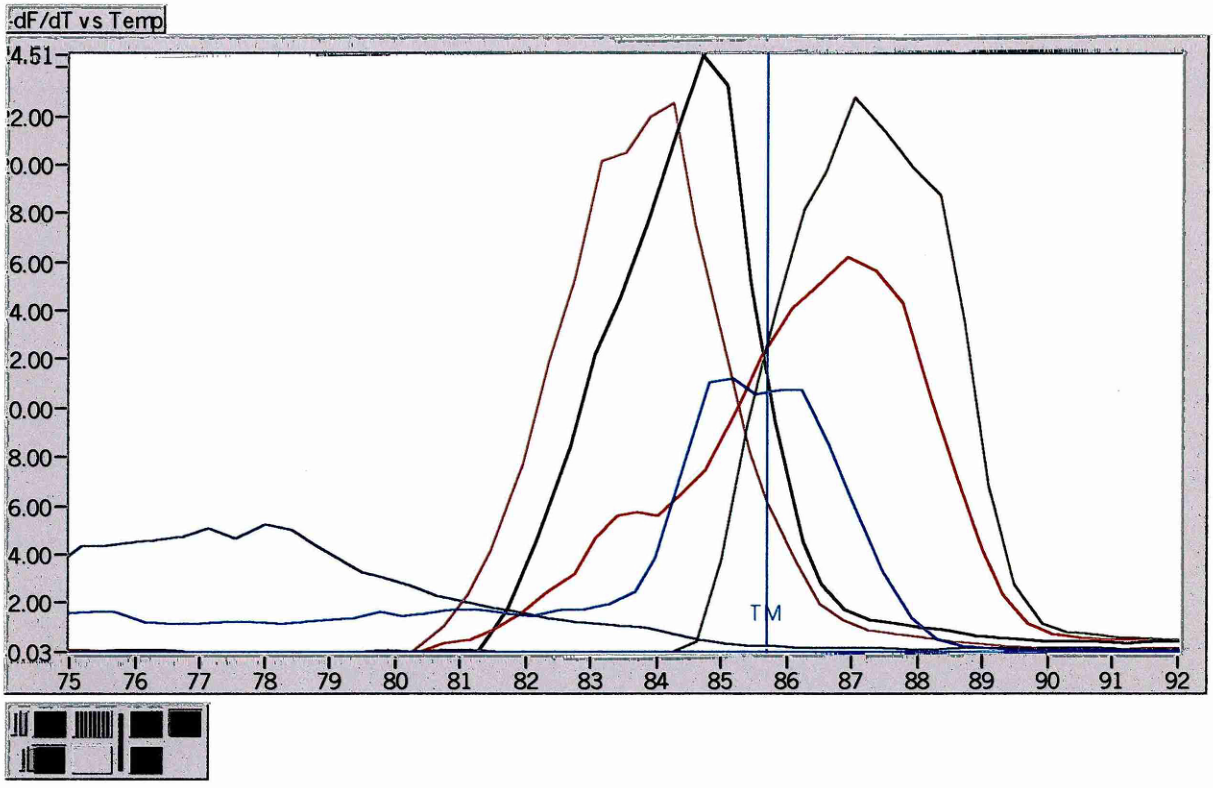
Melting point analysis on the Lightcycler of all five amplicons. Amplification of each template was performed with the individual primer pair with using 0.5µl SYBR-green dilution 1 in 1000, 5µl of forward and reverse primer, 5 µl of LC 3mM MgCl2 buffer (Biogene Ltd) and 2.5 µl of primary PCR product. The cycling conditions were 95°C for 0 seconds, 55°C for 2 seconds, 72 for 10 seconds for 45 cycles (ramp times were 20° per second). Individual peaks represent the melting point of the amplicon. Influenza A H1N1 amplicon is represented as a blue line, influenza A H3N2 is represented as a green line, influenza B is represented as a red line, RSV A is represented as a black line and RSV B is represented as pink line.

Table 3.5 Melting point analysis of each amplicon amplification performed in a multiplex reaction on the Lightcycler

Amplicon	Product size (bp)	Tm of Product (°C)*	% G+C
H1N1	944	85.8	44
H3N2	591	86.5	45
Influenza B	767	86.7	44
RSV A	334	82.7	39
RSV B	183	81	40

The standard Lightcycler amplification annealing time of 2 seconds was not sufficient to amplify all of the five target amplicons. In a multiplex reaction a longer annealing time of 10 seconds was required to achieve amplification of the amplicons (fig 3.24). Products could not be distinguished upon their melting point analysis, as shown in figure 3.24 where the influenza A H3N2 and influenza B products melting point is very similar. Likewise the melting point of the RSV A and B amplicons was too similar to allow differentiation on the Lighcycler analysis software. The Lightcycler was therefore unsuitable for the detection of the multiplex PCR amplicons.

Figure 3.24 Amplification of all five amplicons in a multiplex reaction on the Lightcycler



Multiplex on the Lightcycler of all five amplicons. Amplification of each template was performed with the all primer pairs with using 0.5 µl SYBR-green dilution 1 in 1000, 5 µm of forward and reverse primers, 5 µl of LC 5 mM $MgCl_2$ buffer (Biogene Ltd) and 2.5 µl of primary PCR product. The cycling conditions were 95°C for 0 seconds, 55°C for 10 seconds, 72 for 10 seconds for 45 cycles ramp times were 20° per second).Individual peaks represent the melting point of the amplicon. Influenza A H1N1amplicon is represented as a blue line, influenza A H3N2 is represented as a green line, influenza B is represented as a red line, RSV A is represented as a black line and RSV B is represented as pink line

PCR ELOSA

As an alternative to analysis of the PCR amplicon products by electrophoresis, solid phase detection of them in an ELISA format was investigated. This technique has been called PCR-ELOSA (Enzyme Linked Oligo-Sorbant Assay). Oligonucleotide probes were designed to detect each of the individual amplicons produced from the multiplex PCR (Table 3.6), methods descibed on page 46.

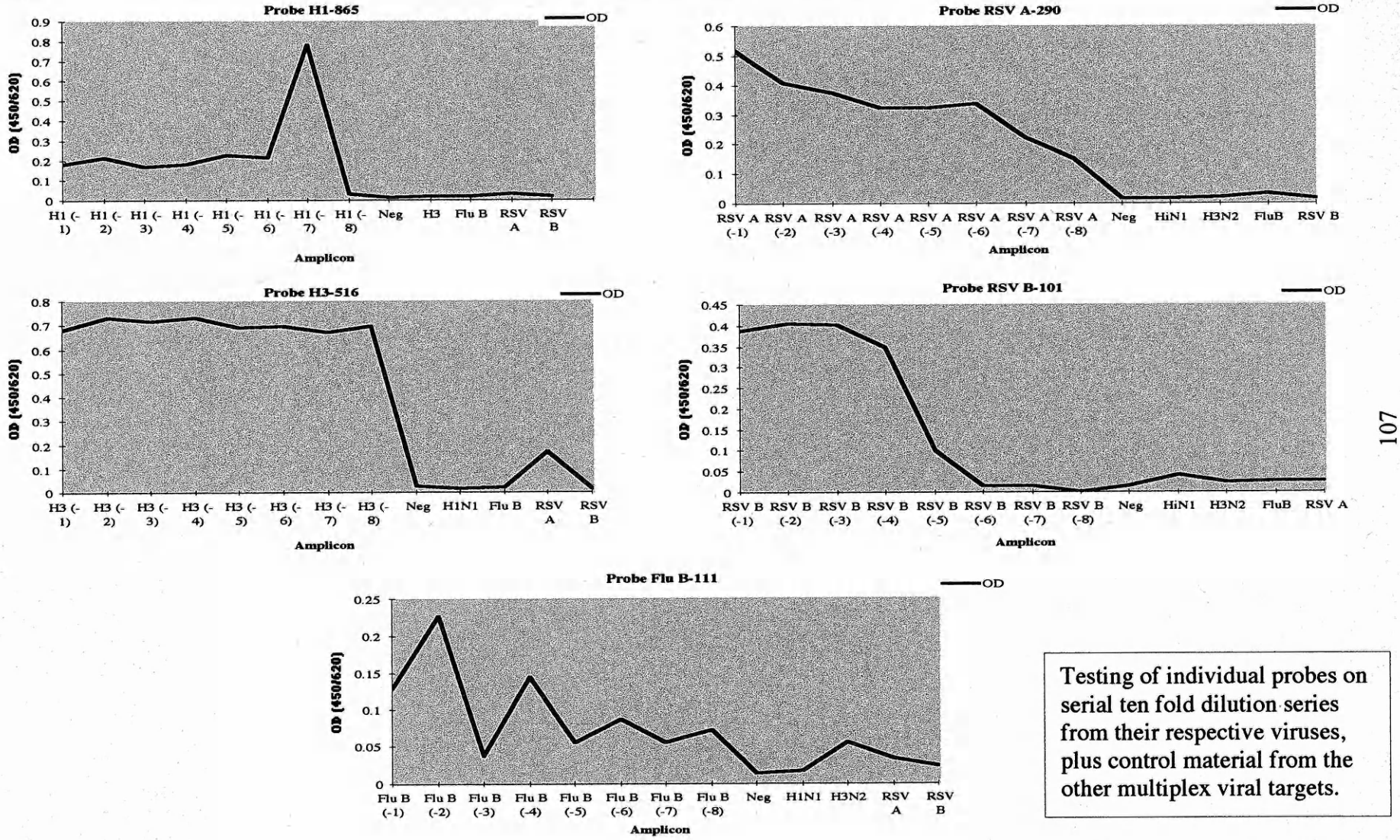
Probes were tested at 100 and 200 ng amounts, with PCR products tested at 1 in 10 and 1 in 5 dilutions. One hundred ng of probe proved sufficient with the PCR amplicons diluted 1 in 5 in hybridisation buffer. Different incubation times were tested with 30 minutes proving the minimum, except for the colour development step which required a minimum of 10 minutes and a maximum of one hour incubation. The initial hybridisation experiments were performed using a water bath; later experiments employed a hybridisation oven to avoid cross-contamination between micotitre plate wells. The RSV A amplicon was found to hybridise to the H3N2 516 probe (fig 3.25), although this was not as efficient as the binding of the H3N2 amplicon to the H3N2 516 probe.

Table 3.6 Probes designed for the detection of the multiplex PCR amplicons in solid phase

Probe	Amplicon template	Amplicon Size (bp)	Nt Position†	5' Spacer	Sequence 5' - 3'	Label 5'	TM °C *	% G+C
H1N1 865	Influenza A H1N1	944	865	aaa	gaatgtacacccagtcagaataggagagtg	Biotin	80.1	46.7
H3N2 516	Influenza A H3N2	590	516	aaa	tgggaaaagctcaataatgaggtcagatgc	Biotin	78.8	43.3
H3N2 140	Influenza A H3N2	590	140	aaa	atgcttgcaaaaggggatctgttaacatt	Biotin	81.5	50
Flu B 111	Influenza B	767	111	aaa	accttcggcacaagcttcaatactccacga	Biotin	81.5	50
Flu B 333	Influenza B	767	333	aaa	cggatttttcgcaacaatggcttgggctgt	Biotin	71.1	34.4
RSV A 290	RSV A	334	2154	ttt	tatgctgaacaactcaaagaaaatggtgtg	Biotin	76	36.7
RSV B 101	RSV B	183	1965	ccc	gtatgcacagaagttgggaggagaagctgg	Biotin	82.9	53.3

*% G + C method (Oligo 5 Software)
† Nucletide position for influenza based on genbank reference strain AF008656, nucleotide psotion for RSV based on genbank reference strain RSH1CE

Figure 3.25 Testing of probes for the detection of the multiplex PCR amplicons in solid phase



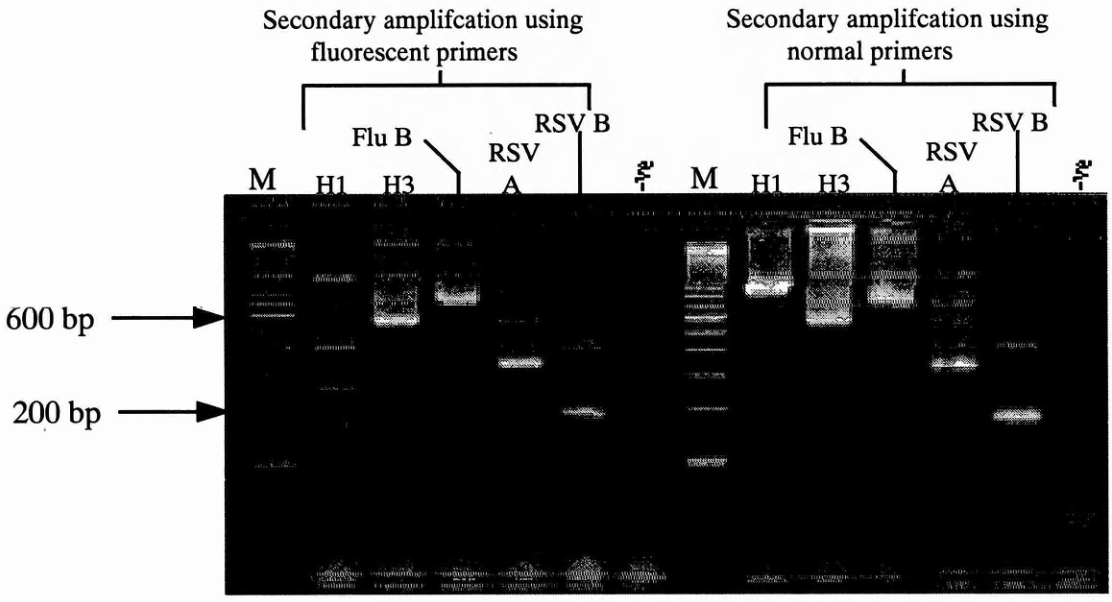
Testing of individual probes on serial ten fold dilution series from their respective viruses, plus control material from the other multiplex viral targets.

Raising the hybridisation temperature from 55°C to 65°C removed the mismatching of the RSV A amplicon to the H3N2 probe. However, it also removed most of the specific hybridisation of the amplicons (results not shown). Lowering the hybridisation temperatures in 1°C intervals from 65°C to 58°C did not result in the specific binding of the amplicons. Mismatching of the RSV A amplicon on the H3N2 516 probe was then investigated *in silico* using OLIGO.

This allowed a different probe (H3 140) to be designed. Testing of H3 140 showed that it did not hybridise to any other amplicon apart from H3N2 amplicon. The influenza B probe (111) intermittently bound the H3N2 amplicon. As more stringent hybridisation temperatures caused a reduction in the specific binding of the other probes, another probe for the influenza B amplicon was designed (influenza B probe 333). Testing of this probe showed that it did not hybridise to any other amplicons and that it gave a strong signal with influenza B amplicon.

It was seen that the H1N1 amplicon only hybridised weakly with the specific probe, producing a low signal. Analysis comparing the amplification of all of the products using both fluorescein bound primers with unlabelled primers showed incorrect amplification of the H1N1 template using the fluorescein bound primers (fig 3.26).

Figure 3.26 Comparison of normal primers and fluorescein labelled primers used to amplify all of the templates from the same primary product.



Amplification of all templates in a multiplex reaction using either unmodified primers or 5' fluorecein labelled secondary reverse primers. Reaction conditions were: 10 mM Tris-HCl pH 8.8, 3.5 mM $MgCl_2$, 25 mM KCl and 1.5 U *Taq* polymerase in both the primary and secondary reactions. M is a marker with base pair sizes indicated with arrows

Discussion

Multiplex PCR assays capable of detecting 13 or more separate regions of human chromosomal DNA, and of detecting and typing several bacterial pathogens have been described by other workers (Appendix 1) (37, 160). However, the use of multiplex RT-PCR for the detection of multiple pathogens with RNA genomes has been much more limited, possibly due to the difficulties of overcoming the inherent inefficiency of the RT step in the RT-PCR or of nucleic acid extraction when the starting material is of poor quality.

The multiplex RT-PCR strategy adopted in this present work was based on the detection of the HA1 portion of the haemagglutinin gene of influenza A and B viruses (70), and on the NP/P region of the RSV genome (fig 3.1). Both of these genomic regions code for antigens used in subtyping of the viruses.

Viral Extraction

The extraction method used in this work involved guanidinium thiocyanate as a chaotropic agent to disrupt the virion and inactivate nucleases and with silica to bind the released RNA (15). This has been shown both to be sensitive for clinical samples containing RNA viruses and very effective at removing inhibitors to PCR which may be contained in the starting material (98).

Primer design

The PCR strategy adopted for the detection and discrimination between RSV A or B was slightly different to that previously described for influenza A and B (70). The primary amplification reaction for RSV uses conserved primers which amplify both RSV A and B (fig 3.1). Subtyping of the virus is achieved in the secondary amplification reaction which uses subtype specific primers. This is in contrast to the influenza virus multiplex PCR, where discrimination between influenza A H1N1, H3N2 and influenza B is at the primary reaction stage with specific primers.

The RSV primers were designed to match as closely as possible, in biochemical and biophysical properties, the primers which had already been designed and optimised for the multiplex RT-PCR-detection of influenza previously developed in this laboratory (70). The parameters considered were the GC content, length and Ta. The main constraint on primer design was the necessity of producing amplicons which could be distinguished on the basis of size, from those already designed for influenza (Table 3.1).

Several different sets of RSV primers were evaluated, both theoretically and experimentally. These included primers located in the F gene, which proved to be unsuitable because of predicted production of mis-primed products in a multiplex reaction, and their empirical failure to amplify template in a multiplex reaction mix. Several problems were encountered during this work which illustrate the difficulty of combining primers in a multiplex reaction. Unexpected interactions between primers (fig. 3.3 & 3.4) impeded the progress of the work, and size restrictions on the resulting product reduced options for subsequent primer choice. A loss in sensitivity of one of the primer pairs (RSV B, fig. 3.7) when they were included in the multiplex was observed. Several methods were tried to overcome the problems encountered (Table 4) with varying degrees of success. Magnesium and *Taq* polymerase concentrations were found to be important for the efficiency of the reaction, as expected (fig 3.10, 3.15). The pH of the tris in the PCR buffer was also found to be important, with any pH less than 8.3 reducing the efficiency of the reaction. This is probably due to the pH decreasing as the PCR progresses, with a too acidic pH becoming inhibitory to the PCR. Another buffer system, the Qiagen Q ammonium buffer (138), was tested but was not found to enhance the sensitivity of the reaction.

The RSV primer sets shown in Table 3.1 were the only ones which were found to amplify both the RSV A and B templates, in a multiplex reaction mix containing all primer sets, to high sensitivity. With the individual templates at high copy number input into the multiplex reaction a smearing was seen on the analytical gel. This is because the test is aimed at detecting virus from low copy number poor quality samples, and so is optimised to provide the maximum possible sensitivity. This is a consideration when designing a diagnostic assay. The conditions used for this multiplex test, and indeed for most in house PCR assays in

diagnostic and reference laboratories, are intended for detection of low copy number targets in poor quality samples. If the assay is intended to be used for detection of virus in high quality and with high copy number samples then other reaction conditions would be appropriate. These would include reducing the sample input volume, reducing the cycle number and hold time, and titrations of the Ta.

Initial evaluation of the sensitivity of detection of equivalent concentrations of RSV A and B RNA templates indicated that amplification of RSV B RNA was substantially reduced in the presence of all primer sets compared to RSV A RNA, and also compared with the use of the RSV B primers on their own. The reason for this could not be determined. However, biochemical optimisation of both the primary and secondary PCR reactions was investigated. This included altering the $MgCl_2$ concentration, buffer formulations, and the buffer pH. Increasing the concentration of *Taq* polymerase in the secondary reaction produced an increase in the intensity of signal of all the amplicons as judged by staining of agarose gels, and subsequent densitometry analysis of the gel result (fig 3.10). The entire optimisation process allowed a substantial increase in the sensitivity of detection of the RSV B RNA until there was an equivalent level of sensitivity of detection of each target RNA in the multiplex reaction (fig 3.15)

Specificity

A wide range of pathogens, both bacterial and viral, may be present in the respiratory specimen samples for which this assay was designed. These will commonly include parainfluenza viruses 1, 2 and 3, enteroviruses, coronavirus, influenza virus, adenovirus, rhinovirus, *Streptococcus pneumoniae, Chlamydia pneumoniae*, *Haemophilus influenzae, Legionella spp* (153). To ascertain whether the presence of other microbial genomes would interfere with the performance of the assay, samples known to contain a variety of pathogens were tested using the multiplex PCR (fig 3.18). No effect could be seen on the performance of the multiplex, and all of the amplicons produced were verified to be the predicted sized and of the predicted sequence (fig 3.5 and 3.17).

Sensitivity

To evaluate the sensitivity of the multiplex PCR for RSV B, it was first necessary to produce a laboratory adapted strain of RSV B which would grow to high titres in tissue culture. This was to allow the serial dilution experiments with all of the targets of the multiplex.

The PCR endpoints were determined by PCRD50 and compared with those measured by infectivity testing in pfu/ml for both influenza and RSV. The PCR procedures employed were very sensitive, much more so than the infectivity tests. In the case of influenza, titration of the sensitivity of detection of purified influenza RNA indicated the ability to detect reliably 40 or fewer genome equivalents of influenza (70). Detection of freshly grown, infectious RSV was at least as sensitive as the detection of infectious influenza in the multiplex reaction mix. It is expected that the sensitivity of detection of a purified RSV RNA template would be approximately equivalent to this, although the virus particle to infectivity ratios may differ slightly between orthomyxoviridae and paramyxoviridae.

Although many reports (135) have shown that including a hot start in a PCR assay can greatly improve the specificity and sensitivity of it, this was not seen in this work (fig 3.16). Inclusion of a hot start to this assay may be of benefit if other polymerases were used. Hot start using a chemically modified enzyme can be extremely expensive and therefore may be not practical to implement when testing large number of specimens.

Final multiplex conditions

The final conditions of 10 mM Tris HCl pH 8.8, 3.5 mM $MgCl_2$, 2.5 mM KCl (Stratagene Optiprime buffer 7) and 1.5 U *Taq* polymerase (Gibco-BRL) were found to be optimal for maximum yield of the specific product of each nested primer set in a multiplex reaction mix. The previously published biochemical conditions (1.5 mM $MgCl_2$, 25 mM KCl, pH 8.8) used for multiplex RT-PCR detection of influenza A and B (70) were found to be sub-optimal for the detection of RSV B (fig. 3.7). The final modifications to the PCR assay protocol included increasing the concentration of *Taq* polymerase in the secondary reaction and increasing the concentration of $MgCl_2$ in both primary and secondary reactions, and altering the buffer conditions. Increasing the concentration of *Taq* polymerase in the secondary reaction

significantly increased product formation (fig 3.10). Lowering the pH of the reaction below pH 8.3 was found to substantially decrease the sensitivity of detection (fig 3.8). The final amplification protocol included an initial denaturation at 94°C for 2 min, then 35 cycles of 94°C for 1 min, 50°C for 1 min and 72°C for 1 min for the primary reaction, and for the secondary reaction a 94°C initial denaturation step for 2 min, then 35 cycles of 94°C for 1 min, 60°C for 1 min and 72°C for 1 min.

The multiplex RT-PCR was tested for specificity on all of the viral targets (influenza A H1N1, and H3N2 and B, and RSV A and B) using first the RSV primers, and then adding each of the influenza primer pairs sequentially, in simulated clinical specimens. No mis-priming was observed when all of the primer sets were present with either a influenza A or B or an RSV A or B template (fig 3.5). A product of the expected size was obtained for each viral template using the multiplex RT-PCR with all of the primer sets present (fig 3.5). The specific products could all be clearly separated and identified on an 2.25% NuSieve agarose gel (fig 3.5). This was observed for both laboratory adapted virus control material (tissue culture grown virus, influenza and RSV, or egg grown influenza) and for clinical samples containing wild type strains. The product specificity of the amplicons obtained from multiplex RT-PCR reactions was also confirmed by sequence analysis. An example sequence alignment is shown in figure 3.17.

Reagent preparation

Preparation of the reagent mix for multiplex RT-PCR containing five primer nested sets is laborious and, in order to investigate whether this part of the procedure could be streamlined, I evaluated whether the preparation of "master mixes" with reagent combinations containing all the necessary primers, buffers and dNTPs could be prepared and stored prior to use. With the use of these, performance of the multiplex PCR would require only the extraction of nucleic acid, addition of it to a pre-prepared tube, and addition of *Taq* polymerase. It was found that storage of pre-prepared reaction mixes did not alter the sensitivity of detection of virus, even when the mastermixes were stored for up to six months (fig. 3.19). Also, no increase in the production of non-specific products was seen with the use of the mastermixes. Thus prior preparation of reagents is feasible, and can increase throughput of specimens for

multiplex RT-PCR. Mastermixes, and the multiplex PCR described here, are now in routine use in the respiratory virus unit.

Multiple template detection

The multiplex RT-PCR reaction was clearly capable of detecting the presence of at least two pathogens simultaneously, in high and low copy number, using all possible combinations of RSV and influenza RNA templates, with both spiked, simulated clinical specimens, and with genuine clinical specimens. The latter case was one where the presence of two viruses had been demonstrated by culture from persistent infection of an immunocompromised child (fig. 3.21). Successful amplification of all five templates in the same reaction mix in a spiked simulated clinical samples required additional *Taq* polymerase in the secondary reaction. There did not appear to be a selective loss of a particular amplicon when the *Taq* polymerase concentration was limiting, as different amplicons failed to amplify in repeat experiments. Failure to amplify a product also did not appear to relate to product size. When the concentration of *Taq* polymerase was doubled for the secondary PCR all five amplicons could be clearly visualised. This suggests that the catalytic action of *Taq* polymerase is a rate limiting factor in achieving simultaneous amplification of multiple templates in the same reaction. However, it is extremely unlikely that an individual would be infected with all five viral targets simultaneously and the concentration of *Taq* polymerase was left at 1.5U in both the primary and secondary amplification steps. This would be sufficient to detect four viral templates in any one sample and was deemed sufficient for this study. Further testing may reveal that the loss of one template was connected with the input copy number in the reaction, with the lowest failing to amplify. This appeared to be the case, with the limited testing conducted in this work, although more extensive experimentation is necessary.

Alternative detection methods

Although agarose gel electrophoresis is the most commonly used method for analysing the results of a PCR amplification, it is not necessarily the most suitable method for all purposes. Therefore, other methods for detection of PCR products were investigated, including real time PCR in sealed glass capillary tubes in the Lightcycler. The second round inner multiplex primers were tested in the Lightcycler. As the outer primers operate at a lower Ta than the

secondary primers, it was not possible to perform a nested reaction within the sealed capillary. Therefore, the primary reaction was performed on a standard thermocycler, and this product transferred to the capillary for testing of the second round primers.
In a standard Lightcyler reaction only a short hold of 2 seconds is commonly used at the annealing stage. When all of the secondary primers of the multiplex (ten in total) were included in the lightcycler reaction, a longer annealing time of 10 seconds was needed to allow amplification to proceed.

Analysis of the multiplex amplicons on the Lightcycler showed that the influenza A H1N1, H3N2 and influenza B had very similar melting temperatures, as did the two RSV amplicons (Table 3.5). Therefore only differentiation between RSV and influenza can be achieved and no subtype information is obtainable with the multiplex PCR in its present format on the Lightcyler (fig 3.24).

However, this is not to say that a multiplex reaction could not be conducted on the Lightcyler. Further experimentation could have involved designing probes capable of distinguishing between the amplicons. However, designing a multiplex reaction for the Lightcycler would have necessitated starting again with the initial primer design. If a multiplex was designed to be performed on the Lightcyler, the design of the amplicons should be such that they have sufficiently different melting temperatures to allow distinction by their melting points. Alternative methods for differentiation, if this proved too difficult, could be explored, including molecular beacons (235, 238, 260).

The most inefficient stage of the process was the necessity for the primary reaction to be performed on an ordinary thermocyler. This was extremely rate limiting. The primary reaction could have been performed on the Lightcyler for a small number of specimens, however the design of the reaction vessel for use in the Lightcycler is such that it was not easily opened once sealed. If this were to be done it would involve a delicate process of unsealing the capillary and spinning the primary reaction out. I believe that for large number of specimens this would have proven to be a major cause of contamination, and would obviously be a time consuming process.

As the clinical samples used in this study are of poor quality and have a low target copy number a nested reaction is required. This is the case for many clinical samples. The speed of cycling that the Lightcycler offers would be extremely advantageous in a diagnostic setting. Careful design of primers would allow a nested reaction to be performed inside the same capillary. If the outer primers were designed to work at a significantly different annealing temperature to that of the secondary primers it may be possible to perform a nested reaction without having to open the capillaries. This would, of course, avoid any contamination and time involved in the primary to secondary reaction transfer.

PCR ELOSA

The detection of PCR products by ELISA type methods (PCR-ELOSA) has been shown to increase the sensitivity of PCR assays in some studies, and the introduction of a probe step has also been described to provide increased specificity (162, 220). The design of the probes for the PCR ELSOA investigated in this work were based upon the amplicons produced in the multiplex. However as PCR amplicons are double stranded DNA, a denaturing step at the beginning of the detection protocol is necessary. Alternatives to heat denauration include asymmetric PCR and chemical means to produce a single stranded DNA product (58, 170). Should an assay be designed using PCR-ELOSA as a method of detection then an asymmetric PCR would be advantageous, as it obviates the need for denaturation of the amplicons (170). This in turn reduces the number of steps in the detection procedure, which will reduce the possibility of contamination occurring. Chemical denaturation is also possible but it requires an additional step which increases the risk of contamination, along with the length of time taken to perform the assay (162, 184).

As this method uses PCR amplicons control of contamination must be rigorously undertaken. Initially, a waterbath was used for temperature control at the hybridisation step. However this resulted in contamination due to the inability to effectively seal the plates. A dry form of heating proved more efficient at maintaining an effective seal on the plates used.

All probes included a non-specific spacer at their 5' end as this has been found to be advantageous (170). The length of the probes was 30bp (excluding the 3 bp non-specific spacer) to ensure that the specificity of the hybridisation would be sufficient to distinguish

between all of the amplicons. In practice shorter probes may have also proved specific, but these were not tested. A problem was encountered with the amplification of the largest template (H1N1) using the fluorescein labelled primers which needs further investigation. It may have been due to the primers themselves, and using a different batch of primers may have allowed correct amplification. Most reports of amplicon detection using this method amplify fragments between 300-500 bp in length (162, 170, 184). This is due to the fact that the probe is only short itself and the longer the DNA bound to the probe may cause it to be less stable. The non-complementary 5' spacer region is believed to aid in the ability of the probe to specifically bind the amplicons, as it allows all of the probe sequences to protrude from the streptavidin support.

The real benefit of this method of detection is, when it is possible, to amplify a generic fragment of DNA (for example ribosomal DNA) and discriminate between different targets solely by use of oligonucleotide probes. This would reduce the need for many primers in the PCR mixture and, in turn, reduce the lengthy optimisation procedures required to assess the primers in combination. PCR-ELOSA also reduces the constraints of primer design as amplicons of specific length are not necessary. However my conclusions from these experiments aimed at converting the multiplex PCR to a PCR-ELOSA format are that it is more complex than originally thought, and therefore it was not pursued. It was more important to have a working and usable multiplex PCR for the subsequent studies than to spend time converting the gel based assay to a robust microtitre plate format.

Chapter 3 Appendix 1

Multiplex PCRs that have been described for detecting human viral pathogens

Target	Nucleic Acid	Number of Pathogens Detected	Reference
HTLV-I and HTLV-II	DNA	2	(248)
HBV	DNA	1	(202)
HIV-1	DNA	2*	(262)
HHV-6 and HCMV	DNA	3*	(168)
Polioviruses and other enteroviruses	RNA	2	(69)
HPV	DNA	3	(263)
Adenovirus and HSV	DNA	2	(120)
HIV-1, HIV-2, HTLV-I and HTLV-II	DNA	4	(112)
HSV-1 and HSV-2	DNA	2	(33)
EBV and (plus *Toxoplasma gondii*)	DNA	2	(206)
HSV and enterovirus	DNA and RNA	2	(32)
Influenza A (H1, H3) and B	RNA	3	(70)
Influenza A (H1, H3), B and C	RNA	4	(264)
HCV and HGV	RNA	2	(34)
HPIV 1, 2 and 3	RNA	3	(68)
HSV and VZV	DNA	2	(12)
Influenza A (H1, H3) B and RSV A and B	RNA	5	(223)
Adenovirus (plus *H. influenzae, M. cataarrhalis, S. pneumoniae*)	DNA	4	(9)
HCMV	DNA	1	(163)
IBV	RNA	2	(244)
HSV-1, HSV-2, VZV and Enteroviruses	DNA and RNA	4	(200)
HSV (plus Treponema pallidum and *Hemophilus ducreyi*)	DNA	3	(204)

- Includes an internal control

Viral Abbreviations

HTLV - Human Lymphotrophic virus, HIV - Human Immunodeficiency Virus, HPV - Human Papillomavirus, HSV - Herpes Simplex Virus, VZV - Varicella Zoster virus, HBV - Hepatitis B virus, HCV - Hepatitis C Virus, HGV- Hepatitis G Virus, HPIV - Human Parainfluenza Virus, EBV - Epstein-Barr Virus, IBV - Infectious bronchitis virus , HCMV- Human Cytomegalovirus, HHV - Human Herpes Virus

Chapter 4

The use of multiplex for the surveillance of community acquired RSV and influenza infections in clinical specimens collected over three years (1995-1998)

Introduction

Every year, during the winter months, hundreds of patients in the UK present to their General Practitioners (GPs) with influenza or influenza like illness (ILI). A subset of practices across the UK partake in a scheme set up by the Royal College of General Practitioners (RCGP) whereby combined nose and throat swabs are collected from patients presenting to them with respiratory illness (79). The symptoms must be severe enough for the GP to suspect influenza as opposed to general 'cold' symptoms. These samples are then sent through the postal system to the Central Public Health Laboratory at Colindale for testing. For the later seasons studied in this thesis the number of participating practices increased, for the 95/96 winter season seven practices participated which increased to 12 in 96/97 and 11 in 97/98.

This scheme was set up primarily to monitor influenza strains in the community, providing information for vaccine updating and an early warning of the circulation of potential pandemic strains. The samples that were collected were tested for influenza in several ways, including culture, ELISA and molecular methods (70, 79). These studies showed that, on average, 40% of the samples sent to the laboratory as part of the RCGP surveillance scheme contain influenza. This in turn means that 60% of respiratory illnesses presenting to GPs with severe symptoms, severe enough for the GP to suspect influenza, are of unknown cause.

England

Nicolson and colleagues (1997) conducted a study in England of the viral infections associated with upper respiratory tract illness (URTI) in elderly persons living in the community (180). They found that rhinoviruses accounted for over half of the positive specimens (52%). Influenza and RSV caused relatively little illness in this group (180). Detection methods used for viral pathogens were complement fixation for specific antibodies, HI for influenza A, and PCR for rhinovirus. As found by Mäkela co-workers (1998) (161), Nicolson *et al* (1997) detected high numbers of rhinovirus infections. In both these studies PCR was used for detection of rhinovirus and was probably more sensitive than the methods used for detection of other viruses.

Similar low detection rates of RSV were found in a study of adults in the UK (158). Culture and serology techniques were used to study samples collected by GPs in one practice from adults with lower respiratory tract infections (158). A total of 206 throat swabs were taken and tested for 17 pathogens. Evidence of bacterial infection was found in 92 (45%) cases, and viral infection in 19 (9%) cases. Influenza was identified in 12 (5%) cases, RSV in 5 (2%) cases with adenovirus and rhinovirus responsible for the rest of the viral cases (158). Identification of RSV in this sample set was low, an explanation for this may be that RSV does not cause severe disease in adults, and therefore does not require a visit to the GP. The finding of Mäkela and co-workers would not support this conclusion (161). The testing methods used were not as sensitive as methods such as PCR for viral detection (70, 223) and it is possible this could account for the low rates of viral detection. Mixed infections were reported in 16 (8%) patients; 13 patients had two pathogens, and three patients had three pathogens. One or more pathogen was detected in 44% of patients (158), which is low in comparison with other studies which tested for both viral and bacterial respiratory pathogens (91, 107, 134).

RSV is a well documented cause of respiratory illness in infants and children (see Chapter 1) and the role of it in infection in other groups of the population, the elderly and hospitalised, has also been described (71, 74). However the role, if any, that RSV has in causing severe respiratory illness in the general adult population is unclear. In order to further investigate RSV as a causative agent in these infections for which influenza is not responsible, the multiplex PCR for influenza and RSV was applied to three seasons of RCGP samples.

Other countries- specific subsets of the population (pneumonia)

Gendrel *et al* studied community acquired pneumonia in French children aged between 18 months and 13 years old (91). Blood samples were tested for RSV, influenza and parainfluenza using a complement fixation assay. Three bacterial pathogens were also tested for: *Chlamydia trachomatis* and *Chlamydia psittaci* were detected by a micro-immunofluorescent test, and *Mycoplasma pneumoniae* was detected by enzyme immunoassay. A total of 104 samples were analysed, 30 were positive for one of the

viruses tested for and 10 were positive for RSV. Mixed infections were found in 8% of the samples, with both bacteria and virus present. No evidence of mixed infections with two types of virus were found, but serological tests for two species of bacteria were positive for one patient. One or more pathogen was identified in 84% of samples (91).

In contrast Heiskanen-Kosma *et al* (1998) found a higher prevalence of RSV (21%) than Gendrel and co-workers (1997) (107). In their study of the aetiology of childhood pneumonia, paired sera were tested for antibodies to several viral and bacterial pathogens. In total, 201 samples from children less than 15 years old in Finland were tested (107). *Streptococcus pneumonia* was the most commonly found organism (28%), whereas RSV was found in 21% of cases and was the most abundant virus found. A mixed infection was identified in 25% of the cases, with two bacterial species identified in 14%, two viral types in 1% and one combined bacterial and viral infection found in 10% of the samples. In total one or more pathogen was identified in 66% of the samples (107). This is less than was found by Gendrel *et al* (1997).

These two studies highlight the role of viral pathogens in the respiratory illnesses of children, with RSV commonly causing a large burden of disease (91, 107). The levels of mixed infections identified in these studies were different, as was the overall level of pathogen identification. These variations may be a reflection of the methods used for detecting the pathogens, or may be influenced by the sample type collected.

The aetiology of pneumonia in Finland was studied in hospitalised adults during an epidemic of *Chlamydia pneumoniae* (134). Here, 125 patients were tested for viruses and bacteria using a variety of serological methods. Their study was of adults in the age range 15-92 years, and the median age of the patients was 66.7 years. No cases of RSV were identified, although 8% of cases had a viral cause with influenza causing half of them. In light of findings from other studies (74, 107, 154), it is suprising that no RSV was found in these samples, however this may be due to the type of sample and the tests used as this study was mainly aimed at bacterial identification. Mixed infections were identified in a high percentage of samples (38%). Two pathogens were identified in 31% of samples, three

pathogens were identified in 6% of patients and four pathogens were identified in 1% of patients (134). The majority of mixed infections were with two or more bacterial pathogens. One or more pathogen was identified for 88% of samples (134). This level of pathogen identification was comparable with that found by Gendrel and colleagues, although the populations studied were different.

Other countries- general community

Studies have been conducted elsewhere in the world with the aim assessing the causes of respiratory illness. Lina *et al* (1996) studied respiratory viral illness during a winter season in the Rhones Alpes region of France (154). Nasal swabs were taken from patients presenting to their GP with acute influenza like illness. The respiratory viral pathogens tested were RSV, influenza, coronavirus and rhinovirus. Of the 962 samples studied, 36% were positive for one or more virus. RSV and influenza caused similar amounts of infection at 11% and 12% respectively. The majority of RSV infections (83%) were in children (less than 15 year old), whereas only 58% of the samples were from children. The RSV season ran from week 46 to week 10 with peak activity in week 2 in agreement with other studies from this part of the world (91). A significant correlation was found between RSV and bronchiolitis (154). RSV was detected by tissue culture cytopathic effect (CPE) monitoring and immunostaining. Some samples were hand carried to the laboratory and some were sent through the post. The levels of RSV in the community were found to be high, but this was mainly in children. Mixed infections (1.7% of total specimens) were identified predominately in the less than 10 year olds, with 36% of samples having one or more pathogens (154). This is low in comparison with the previously discussed studies (91, 107, 134) however, in these studies samples were tested for both bacterial and viral pathogens.

In comparison to the results of Lina *et al* (1996), a study conducted in southern Israel on the aetiology of respiratory tract infection in adults found relatively few RSV infections (1.6% of total samples were positive for RSV) (153). In this study 122 patient samples were collected from a general practice setting and were analysed for 17 pathogens, both viral and bacterial by serological methods. The age range of patients was 21-78 years, with a mean age of 44.8 years. Bacterial pathogens were found in 43% of patients. *Chlamydia*

pneumoniae was the most common. Viral pathogens were found in 35% of patients and by far the most common was influenza, causing 31% of infections. Mixed infections were identified in 34% of the patients, with a mixed infection of viral and bacterial aetiology in 11% of patients. A mixed infection with two types of influenza virus was reported in three (2.5%) patients (153). In total pathogens were detected in 66% of the samples. One reason for the low detection levels of RSV may be that the samples were collected between January and March. The RSV season may have started earlier than this, and therefore been missed.

Two reasons for the relatively few reported cases of RSV are either that patients with RSV do not have severe enough illness to present to their GP, or that the methods used to detect RSV are insensitive. In a study of the etiology of the common cold in Finland very little RSV (2%) was found (161). Samples were collected between October 1994 and November 1995 from 200 young adults with self-diagnosed common cold symptoms. Samples were tested for 17 pathogens, both viral and bacterial. Methods for testing included serology, antigen detection, isolation and PCR, which was only performed for rhinovirus detection. Viral etiology was identified for 69% of infections with the most commonly virus found being rhinovirus, which was identified in over half the total number of samples. Bacterial infections were identified in only seven patients, and six of those were associated with a mixed infection with a virus (161). Detection of influenza was also at low levels (6%), with mixed viral infections at 5% (total mixed infection 8%) (161). It is interesting to note that virus culture for rhinovirus detected 80 positive samples, whereas PCR detected 103 positive samples, highlighting the differences in sensitivity of detection between the two methods (161). Importantly these results do not support the hypothesis that RSV infection in adulthood causes mild cold like symptoms. If this were the case expected levels of detection for RSV would be higher in studies, like this one, of aetiology of the common cold (108, 161). The detection methods used in this study may not have been the most sensitive, but they are comparable to methods used in other studies (153).

Although high levels of mixed infection seem to be associated with bacterial pathogens it is impossible, from these studies, to ascertain whether both pathogens are contributing to disease symptoms. It is also difficult to know whether disease associated with two or more pathogens is more severe than disease associated with one pathogen. Likewise, it is impossible to determine whether disease symptoms associated with a combined bacterial and viral pathogen are more or less severe than those associated with two or more bacterial pathogens or with two viral pathogens.

Aims of this part of the work

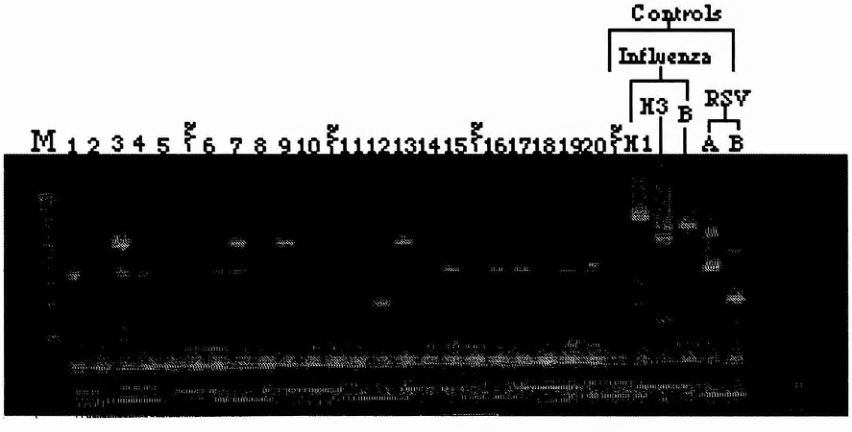
To determine the extent to which RSV contributes to respiratory disease in the general community in England and Wales during the winter months, the multiplex PCR was applied to 2, 235 combined nose and throat swabs. Swabs were collected by a sentinel GP scheme (RCGP) from any person presenting with influenza or ILI in the general community during three winter seasons (95/96, 96/97 and 97/98). Samples taken from the 95/96, 96/97 and 97/98 seasons were stored at -70°C after testing for influenza. Stored cDNA, which had previously been tested, was available for the majority of the 95/96 and 96/97 seasons. Samples were freshly extracted for the 97/98 season.

Results

For this part of the work RNA was extracted from the clinical specimen, reverse transcribed into cDNA and amplified by multiplex PCR with the methods described in Chapters 2 (Materials and methods) and 3 (Design, optimisation and validation of a multiplex PCR). The PCR products were analysed by agarose gel electrophoresis and the results recorded in an Excel database.

A typical gel image of the results obtained throughout this work is shown in fig 4.1. Negative controls were placed after every five specimens and the positive controls at the end of each run. For example, RSV A positive specimens are seen in lanes 1, 3, 4, 5, 6, 15, 16, 17, 19 and 20, a dual RSV A and influenza A H3N2 specimen seen in lane 3 with a RSV B positive specimen seen in lane 12 and influenza H3N2 positive specimens seen in lanes 7, 9 and 13.

Figure 4.1 A typical gel image seen throughout this study

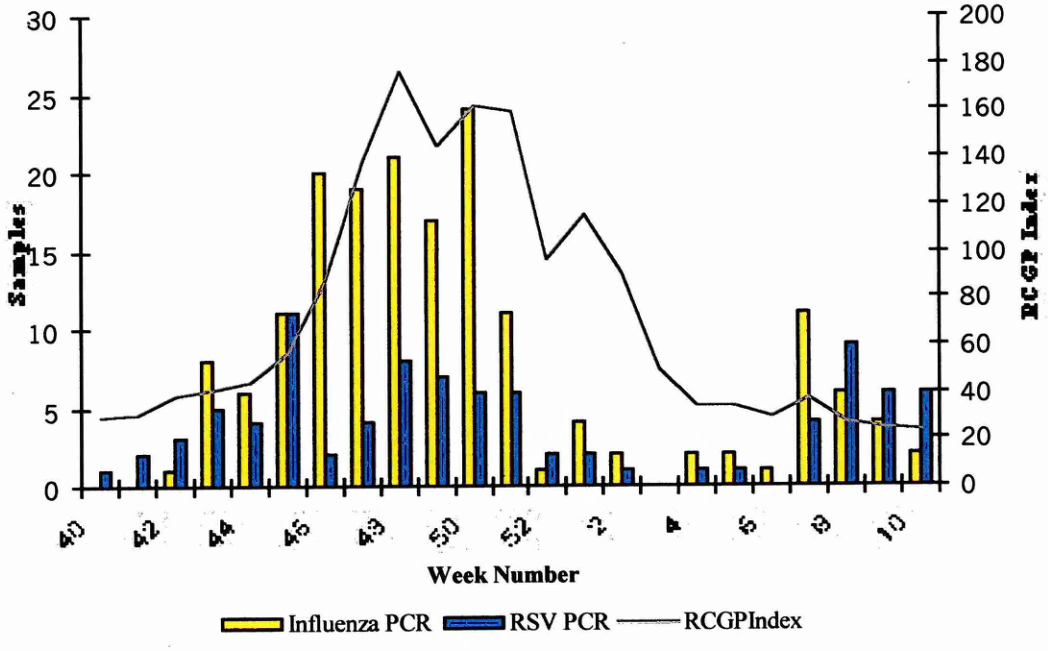


M represents a marker with the bp sizes indicated with arrows. Lanes 1to 20 are combined nose and throat swabs collected via the RCGP network. Positive and negative controls are indicated

Winter season 1995/96

A total of 619 of combined nose and throat swabs were tested by multiplex PCR and infectivity assay. Week by week analysis of the distribution of influenza and RSV throughout the season is shown in fig 4.2. The RSV season started in week 40, two weeks earlier than the influenza season. Week 45 had the highest number of RSV positives, equivalent to influenza for that week. The RSV season appeared to have two main peaks of activity, the first from week 40 to 51, the second from weeks 7 to 10. Weeks 52 to 6 only had a few RSV positive cases. This was a similar pattern to that observed with influenza. The tail end of the RSV season showed a higher number of cases than influenza for the corresponding weeks. For influenza, the main peaks of activity followed the same pattern as the RCGP consultation rate for influenza or ILI.

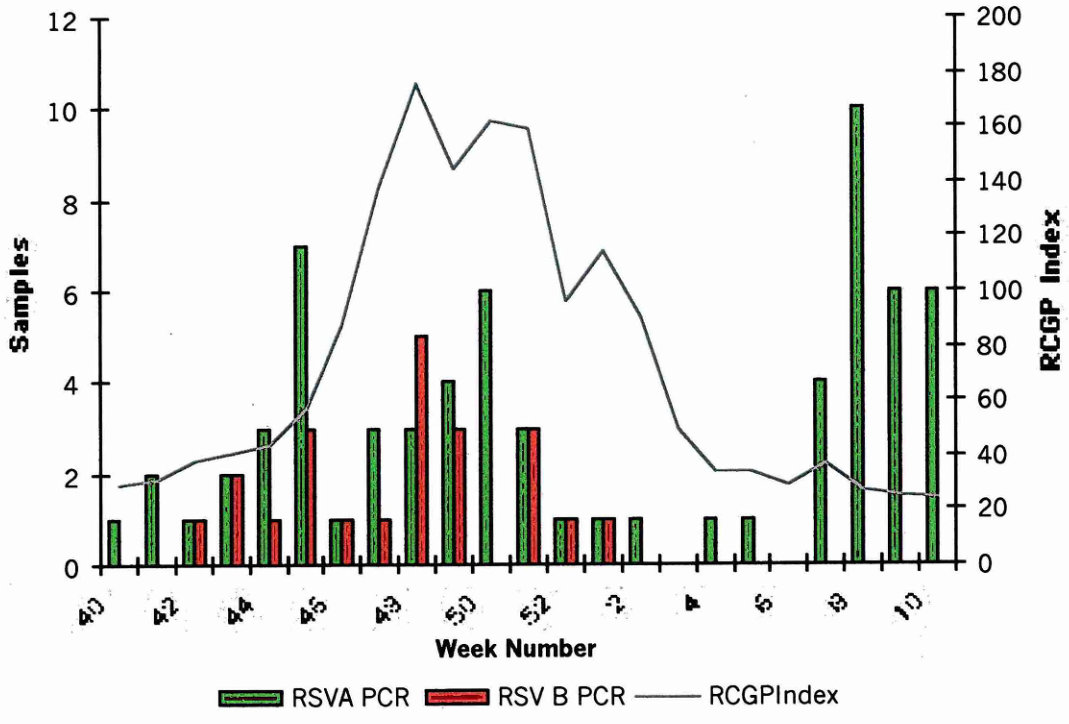
Figure 4.2 Week by week distribution of RSV and influenza as identified by multiplex PCR (95/96 winter season)



The RCGP index represents the consultation rate per 100, 000 of the UK population for influenza or influenza like illness (ILI).

Analysis of the RSV A and B subtypes distributed week by week (fig 4.3) showed that RSV A was the dominant subtype throughout the season. The RSV B season started a little later than the RSV A season at week 42 and ended in week 1. The RSV A season started at week 40 and ended at week 10. RSV A showed two main peaks of activity, with the tail end of the season having a higher number of than at the beginning of the season. The peak of RSV activity did not co-incide with the RCGP index.

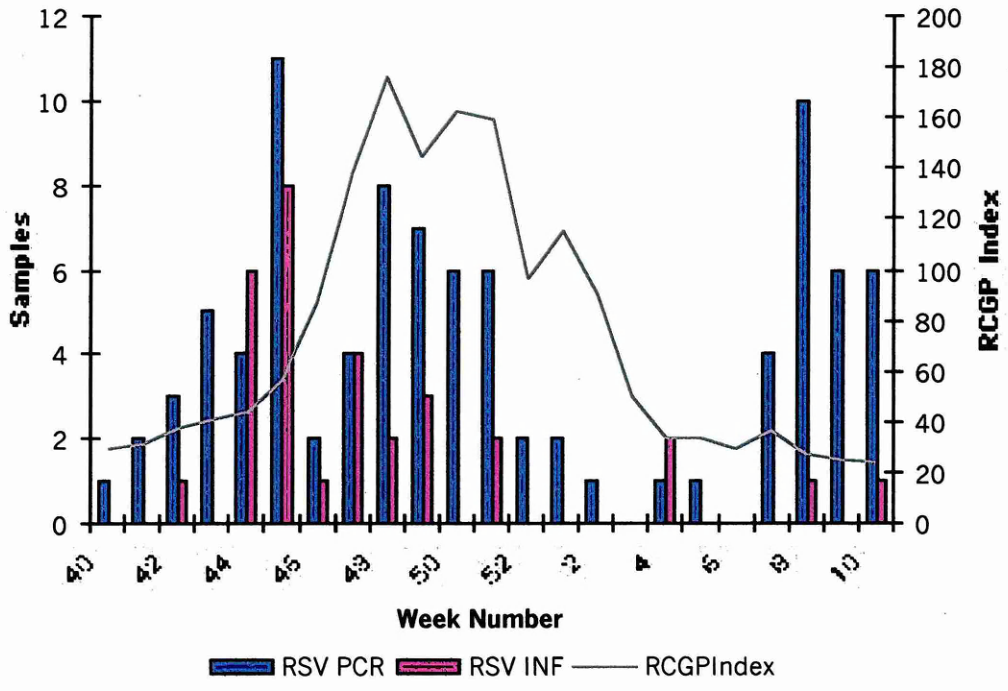
Figure 4.3 Week by week breakdown of subtypes of RSV as identified by multiplex PCR (95/96 winter season)



The RCGP index represents the consultation rate per 100, 000 of the UK population for influenza or influenza like illness (ILI).

A comparison of the detection of RSV by infectivity assay and multiplex PCR is shown as a week by week breakdown in fig 4.4. Overall the correlation with the infectivity assay was poor (52% concordance). The multiplex PCR was more sensitive than the infectivity assay and identified 98 positive samples, whereas the infectivity assay detected 31 positive samples. In week 44, however, the infectivity assay found two more positive samples than the multiplex. Also in week 4 the infectivity assay found one more positive sample than did the multiplex PCR.

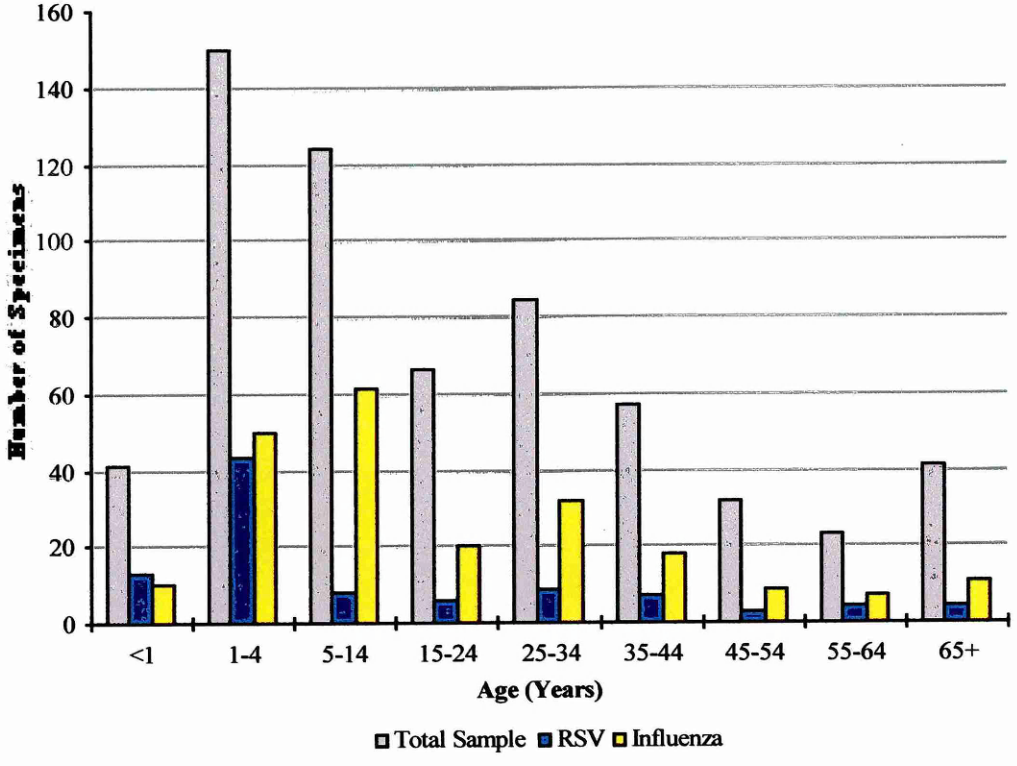
Figure 4.4 Week by week breakdown of RSV PCR compared with RSV Infectivity assay results (95/96 winter season)



The RCGP index represents the consultation rate per 100, 000 of the UK population for influenza or influenza like illness (ILI).

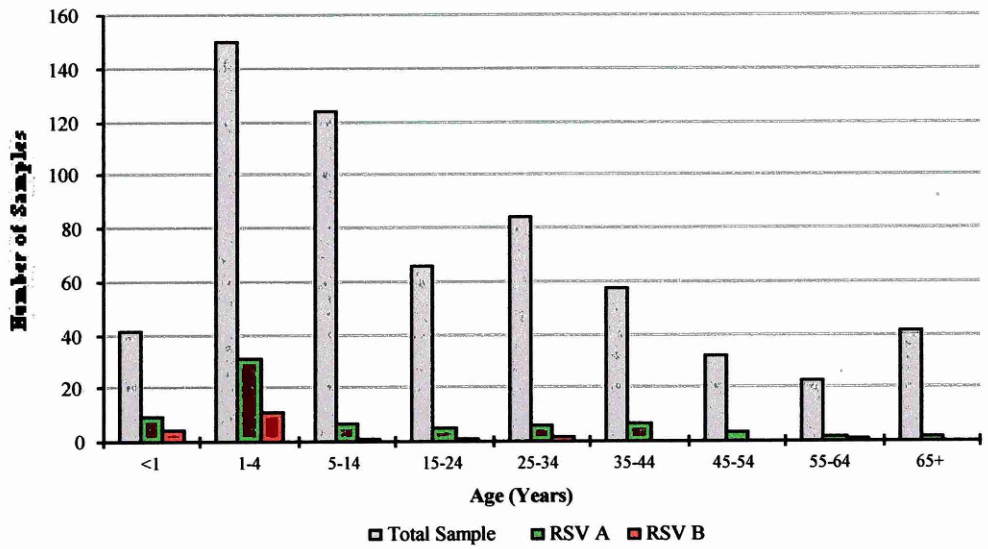
The age distribution of the patients having an RSV infection is shown in fig 4.5. The age range of the patients were from less than one month to 85 years, with a mean age of 21.7 years. Of the total samples, 51% were from children (less than 15 years old), with almost half of these in the age range 1 to 5 years.

Figure 4.5 Age distribution of influenza and RSV infections as identified by multiplex PCR (95/96 winter season)



The age breakdown for patients with either a RSV A or B infection is shown in fig 4.6. RSV B was observed to cause a significant proportion of infections in the five years old and under age range, but only accounted for an occasional isolated infection in the older age ranges. In contrast RSV A caused infections in all of the age ranges.

Figure 4.6 Age breakdown of the RSV positive patients correlated with RSV subtype (95/96 winter season)



The analysis of the total burden of illness caused by RSV and influenza virus is shown in Table 4.1. Overall RSV accounted for half the number of infections that influenza accounted for, with the predominant subtype being RSV A. The sex of the patients from whom the specimens came is shown in Table 4.2. There was no difference in the viral infections identified in either sex.

Table 4.1 Percentage burden of illness caused by RSV and influenza out of the total samples (95/96 winter season)

	Total	RSV	RSV A	RSV B	Influenza
Number of Samples	619	97	73	21	218
(% of total)		15.7	11.8	3.4	35

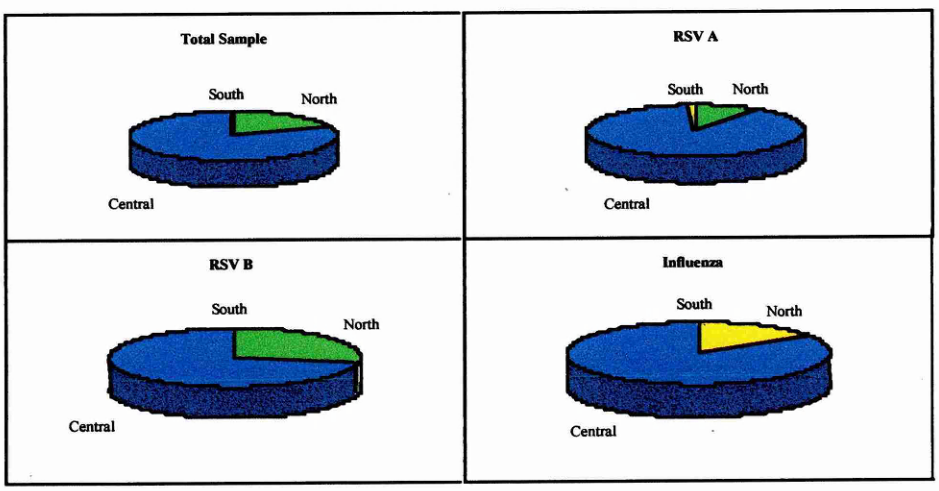
Table 4.2 Sex distribution of patients with a RSV infection (95/96 winter season)

Sex	RSV A	RSV B	RSV A+ B	Influenza	Total Specimen
Male	38	10	2	114	309
%*	12	3	<1	37	
Female	36	10	1	114	310
%*	12	3	<1	37	

* Percentage of the total for that group

The geographical distribution of the samples, influenza virus and RSV infections is shown in fig 4.7. The largest number of samples was from the central region of the UK, and this is reflected in the distribution of RSV and influenza infections. Only two samples were from the southern region and influenza was detected in both.

Figure 4.7 The geographical spread of number of samples correlated with influenza and RSV infections (95/96 winter season)

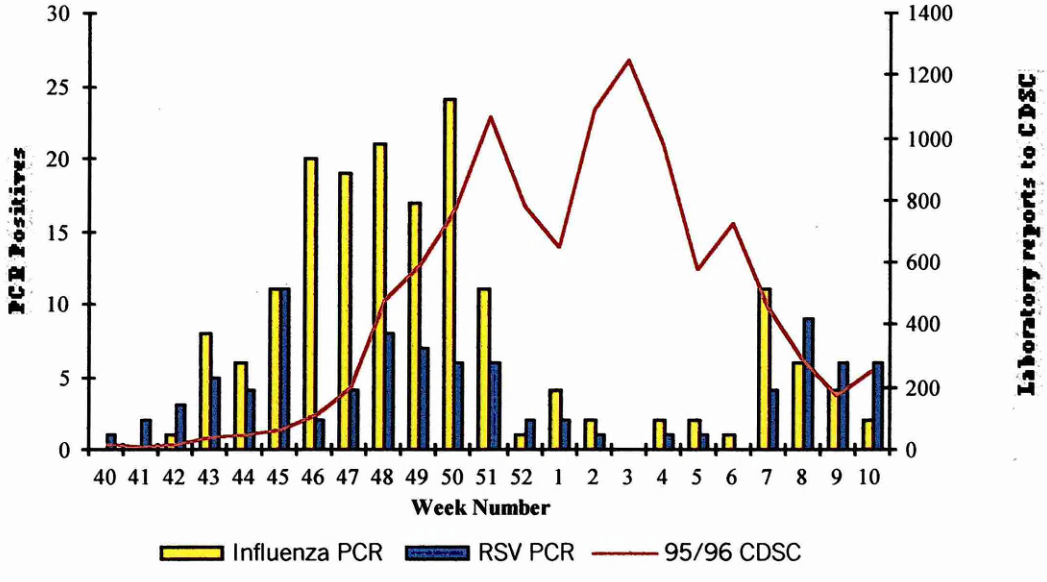


Colour Key

Central = blue, South = yellow, North = green

The Communicable Disease Surveillance Centre (CDSC) London collates data from hospitals around the country based on the laboratory confirmed reports of RSV infection. A comparison of the data from the RCGP ILI sample analysis and the CDSC figures is shown in fig 4.8. The peak activity reported by the two analyses do not correlate, with the CDSC reports of peak activity occurring during the decrease in activity as determined by the RCGP sample analysis.

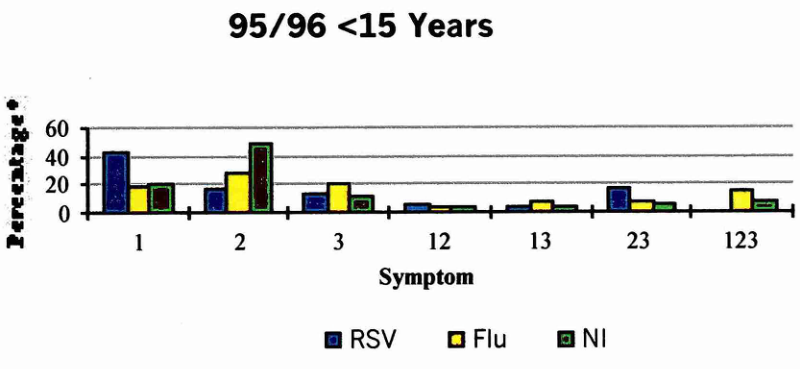
Figure 4.8 Analysis of RCGP results compared with CDSC reports of RSV infections (95/96 winter season)



Laboratory reports to CDSC are of confirmed cases of RSV, mainly from children, from hospitals throughout the UK

For the RCGP analysis symptoms were recorded in three categories by the GP, on a data sheet that was sent, and returned, along with the sample for analysis. Upper respiratory tract infection (URTI), lower respiratory infection (LRTI) and constitutional illness (fever) was scored by the GP in one or a combination of these categories. Analysis of symptoms for the under 15 years old age range is shown in fig 4.9. In this age bracket there were 63 RSV positive samples, 119 influenza positives, and 141 for which no viral pathogen was identified (NI). The largest number of the RSV positives was associated with URTI (43%), whereas the largest number of the influenza positives was associated LRTI (28%). However no viral pathogen was identified in a large number of LRTI cases.

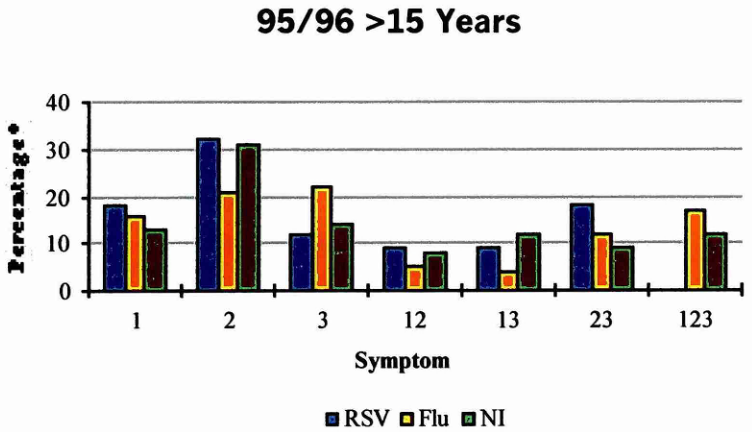
Figure 4.9 Symptom analysis: Individuals under 15 years old (95/96 winter season)



Key to symptom category's scored by the GP upon collection of the sample
1- URTI 2-LRTI 3-Consitutional
12-URTI & LRTI 13-URTI & Constitutional 23-LRTI & Constitutional
123- URTI & LRTI & Constitutional

Analysis of symptoms for the over 15 years old age range is shown in fig 4.10. For this age group there were 34 RSV positive samples, 99 influenza positive, and 163 samples for which no pathogen was identified. In comparison with the under 15 years old age range, the majority of RSV positive patients presented with LRTI (32%). For the influenza positive patients many presented with URTI, or LRTI, or fever, or a combination of all three. For the samples for which no viral pathogen was identified the majority of patients presented with LRTI (31%), as was found for the under 15 years old age range.

Figure 4.10 Symptom analysis: Individuals over 15 years old (95/96 winter season)



Key to symptom category's scored by the GP upon collection of the sample
1- URTI 2-LRTI 3-Consitutional
12-URTI & LRTI 13-URTI & Constitutional 23-LRTI & Constitutional
123- URTI & LRTI & Constitutional

A total of 17 dual infections was identified during the 95/96 winter season, and analysis of these is shown in Table 4.3. Of them, 14 (82%) were an infection with an influenza subtype and a RSV subtype. There were no dual infections identified which involved two influenza subtypes. In contrast two infections were with the two RSV subtypes. No infections were identified which involved more than two viruses. Of the dual infections, 11 were in children (less than 15 years old) and six in adults. The symptoms associated with the dual infections were the same as those associated with single infections, but the severity of illness was not recorded.

Table 4.3 Dual infection reports (95/96 winter season)

Dual Infection Type	Number of Samples
Influenza A H1N1 & RSV A	6
Influenza A H3N2 & RSV A	2
Influenza A H3N2 & RSV B	5
Influenza B and RSV A	1
RSV A & B	3
Total Dual	17

A summary of the 95/96 season is shown in Table 4.4, a total of 315 samples (51%) had a viral pathogen identified.

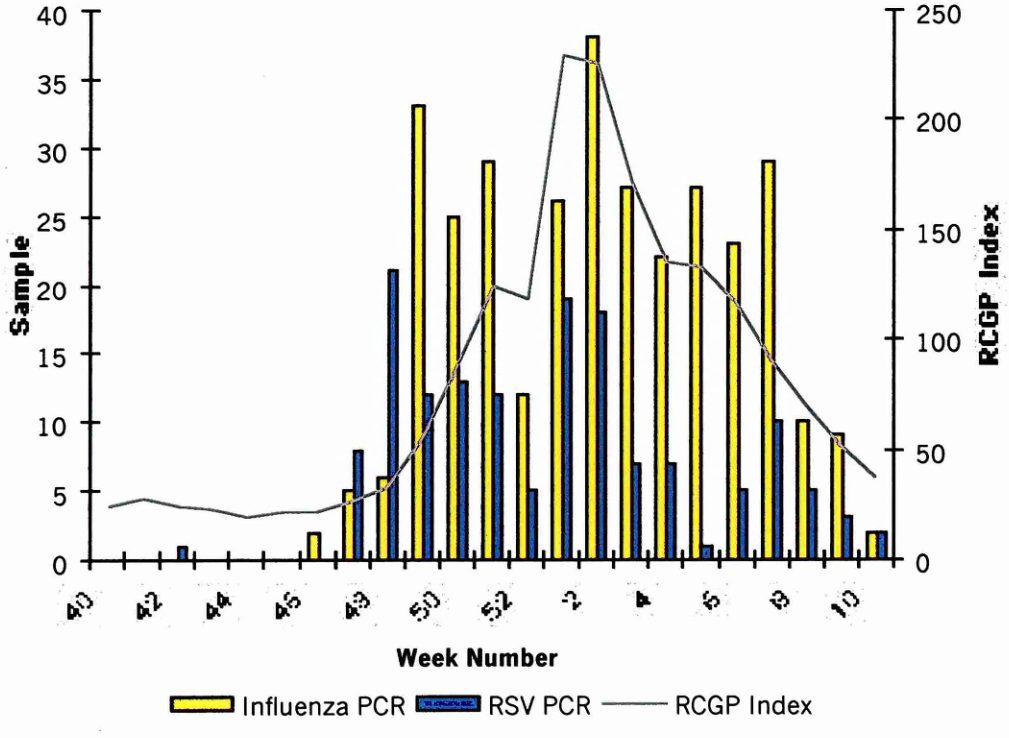
Table 4.4 Summary of data for the 95/96 winter season

	Total	RSV	Influenza	Dual
Number of Samples	619	97	218	17
% (of total)		15.7	35	2.7

Winter season 1996/97

A total of 809 combined nose and throat samples were tested. Week by week analysis of influenza and RSV activity throughout this season is shown in fig 4.11. The RSV and influenza began to be detected in week 45, apart from one outlying RSV positive sample in week 42. The RSV season showed two main peak activity times, although this was not as pronounced as the 95/96 season. The RSV activity peaked in week 48, and remained high until week 2, after which the activity declined until week 6 when the incidence of RSV infections increased again. The influenza season began in week 46, with peak activity seen in week 2. Both RSV and influenza followed the same pattern as the RCGP consultation rate for influenza or ILI.

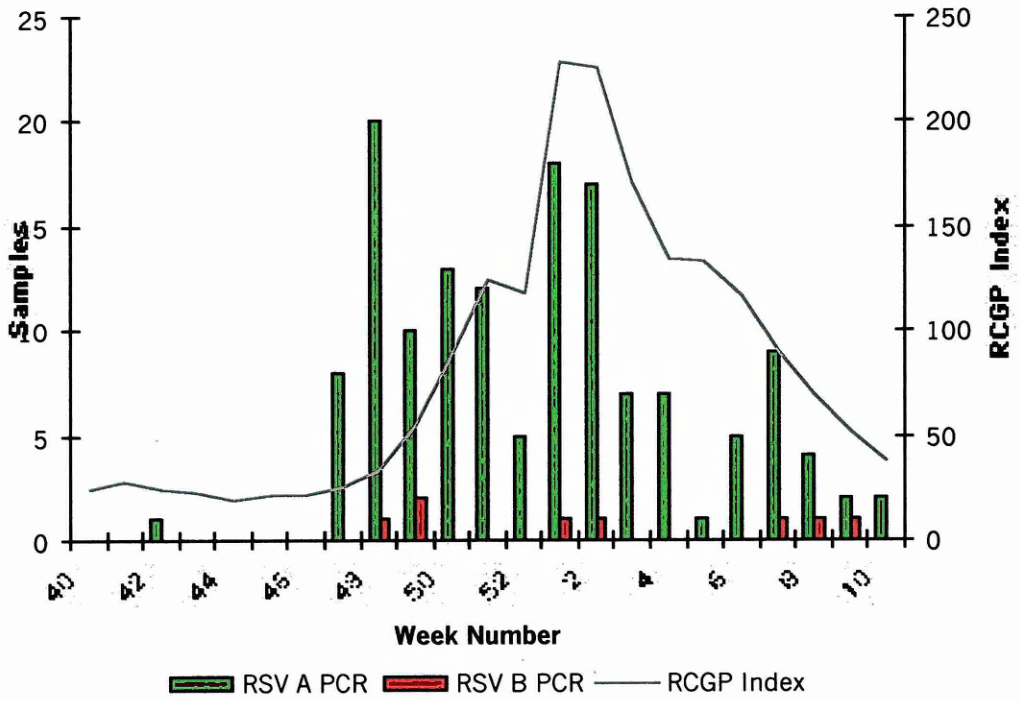
Figure 4.11 Week by week distribution of RSV and influenza infections identified with the multiplex PCR (96/97 winter season)



The RCGP index represents the consultation rate per 100, 000 of the UK population for influenza or influenza like illness (ILI).

RSV A was the predominant subtype of RSV circulating throughout the entire season. One RSV A infection was seen in week 42; the season started in week 47 and ended in week 10 (fig 4.12). The RSV infections were seen from week 48 to week 11. The few RSV B infections which were seen were scattered throughout the season. Although the peak activity for RSV was week 48, and the RCGP index peaked in weeks one and two, the general pattern was the same for the sets of data.

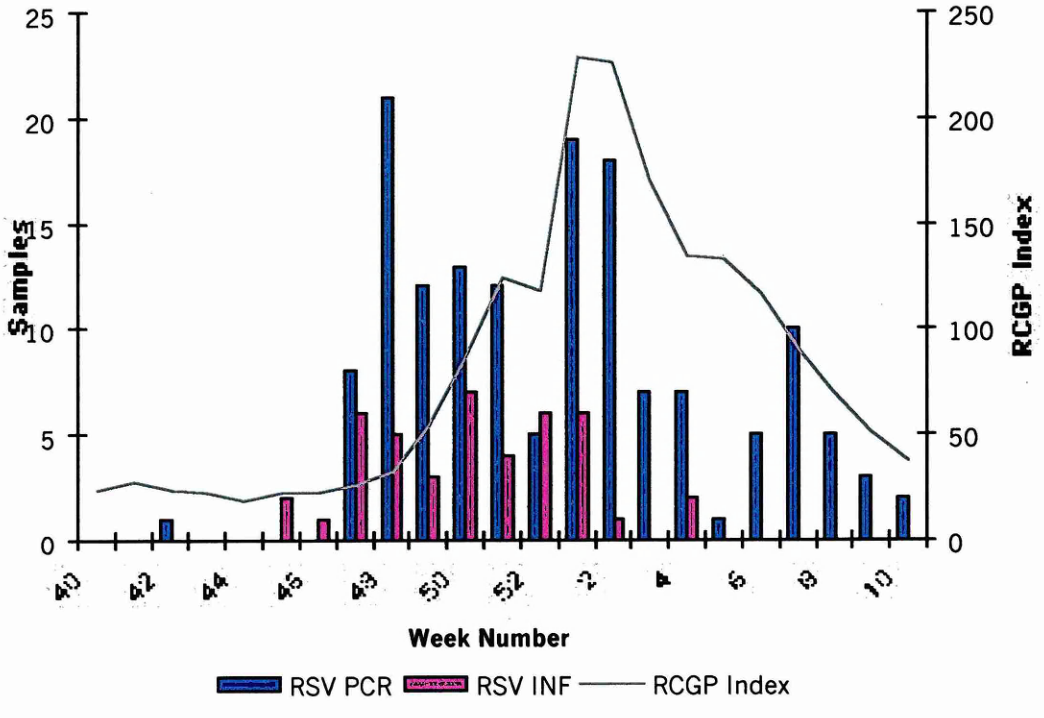
Figure 4.12 Week by week breakdown of subtypes of RSV identified with the multiplex PCR (96/97 winter season)



The RCGP index represents the consultation rate per 100, 000 of the UK population for influenza or influenza like illness (ILI).

The infectivity assay was performed on samples collected until week 6. A comparison of these results with those from the multiplex PCR is shown in fig 4.13. Overall the multiplex PCR was found to be more sensitive than the infectivity assay, and there was poor correlation between the results, with only 40% of the infectivity assay positives also detected by the multiplex PCR. The multiplex identified 187 positive samples whereas the infectivity assay identified 50 positive samples. The infectivity assay identified three infections in weeks 45 and 47 which the multiplex PCR did not identify.

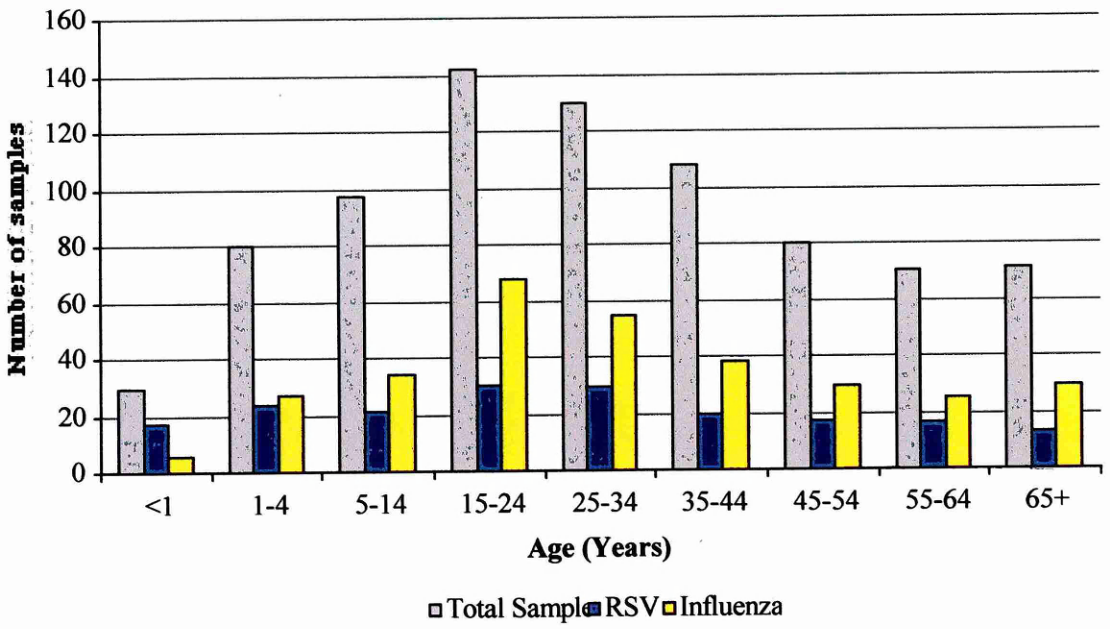
Figure 4.13 Week by week breakdown of RSV PCR compared with RSV Infectivity assay results (96/97 winter season)



The RCGP index represents the consultation rate per 100, 000 of the UK population for influenza or influenza like illness (ILI).

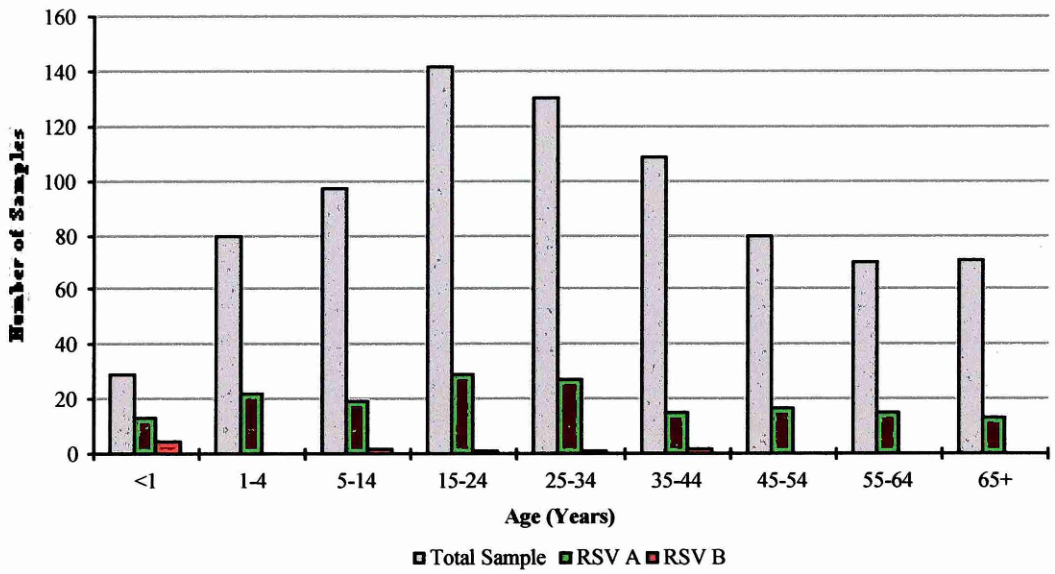
RSV infections were seen to occur in all of the age ranges sampled (fig 4.14). The age range of the patients from whom the samples were collected was from less than one month to 97 years old, with a mean age of 31.7 years. Of the total samples, 25% were from children (under 15 years old), in contrast to the 95/96 winter season. For the under one year old age range the number of RSV infections was over twice that of influenza. For the one to five years old age range the number of RSV and influenza infections were roughly equivalent. For all of the other age ranges the number of RSV infections was at least half that of influenza infections.

Figure 4.14 Age distribution of influenza and RSV infections (96/97 winter season)



RSV A was observed to cause disease in all the age ranges tested (fig 4.15). The few RSV B infections were also spread throughout different age groups (fig 4.15).

Figure 4.15 Age breakdown of the RSV positive patients correlated with RSV subtype (96/97 winter season)



An analysis of the total burden of illness associated with RSV and influenza is shown in Table 4.5. RSV overall accounted for over half of infections that influenza did, with the predominant subtype being RSV A.

Table 4.5 Percentage burden of illness caused by RSV and influenza (96/97 winter season)

	Total	RSV A	RSV B	Total RSV	Influenza
Number of Samples	809	170	10	187	311
% (of total)		21	1	23	39

The sex distribution of the patients from whom the samples were collected is shown in Table 4.6, and infections with RSV and influenza virus were approximately evenly distributed between men and women

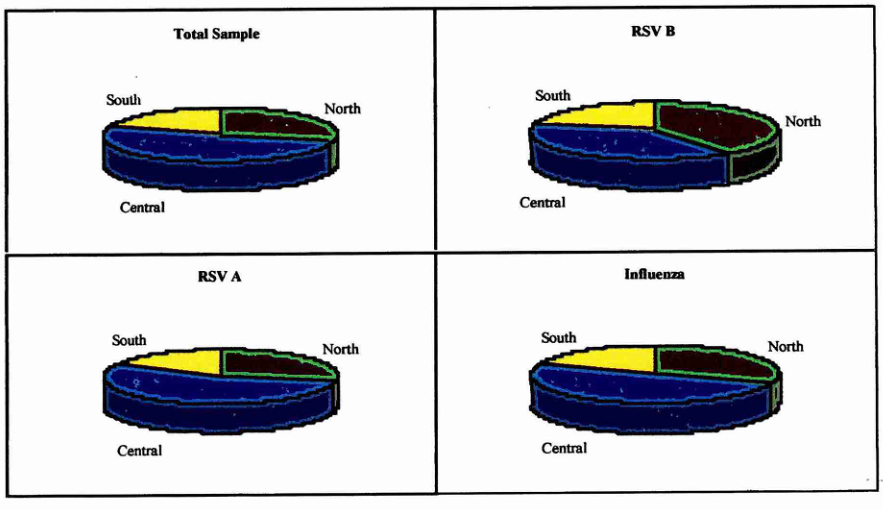
Table 4.6 Sex distribution of patients with RSV infections (96/97 winter season)

Sex	RSV A	RSV B	RSV A+ B	Influenza	Total Specimen
Male	79	8	3	151	385
%*	10	1	0.4	19	48
Female	91	2	4	160	424
%*	11	0.1	0.5	20	52

*% of total for that group

The majority of samples were from the central region of the country, although these were more from the north and south than in the 95/96 winter season. The geographical distribution for each virus was observed to be similar to the distribution of them in the total sample set (fig 4.16).

Figure 4.16 The geographical spread of numbers of samples correlated with influenza and RSV detection (96/97 winter season)

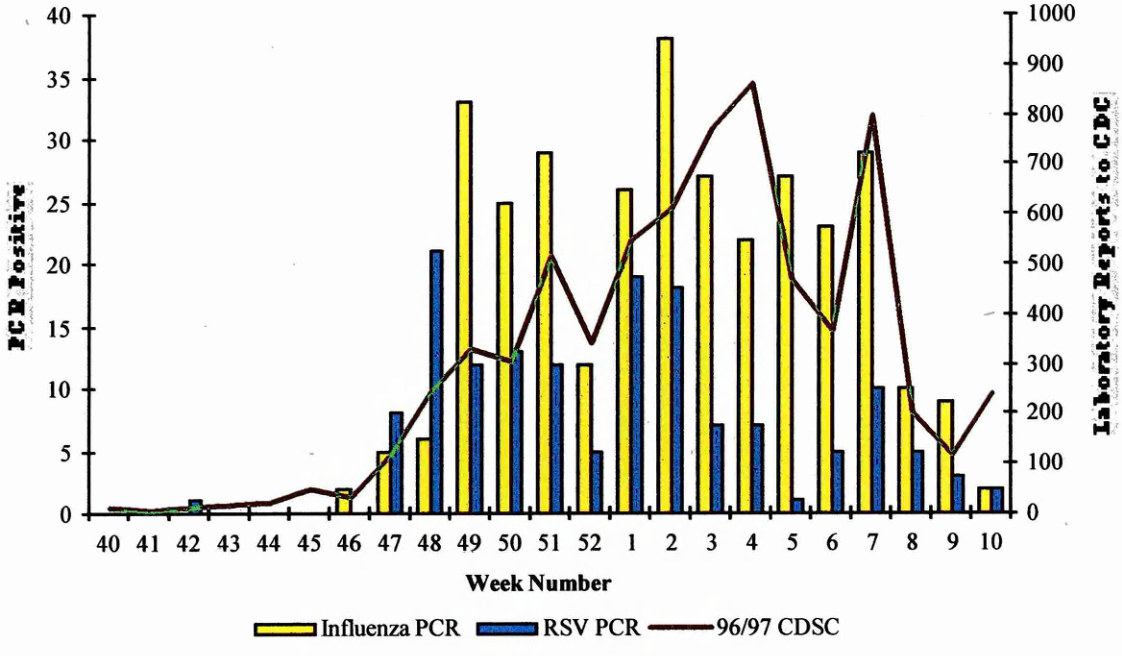


Colour Key

South = yellow, North = green, Central = blue

A comparison of the data from the RCGP scheme (monitoring ILI) and the CSDC RSV analysis is shown in fig 4.17. The CSDC reports of RSV did not peak at the same time as the RCGP scheme. The first peak of activity for RSV from CSDC reports was seen in week 4, whereas the same week in the analysis of the RCGP data showed a decline in ILI. However there may be a delay in reporting of infections to CDSC and the general pattern seen with the two analyses are similar (fig 4.17).

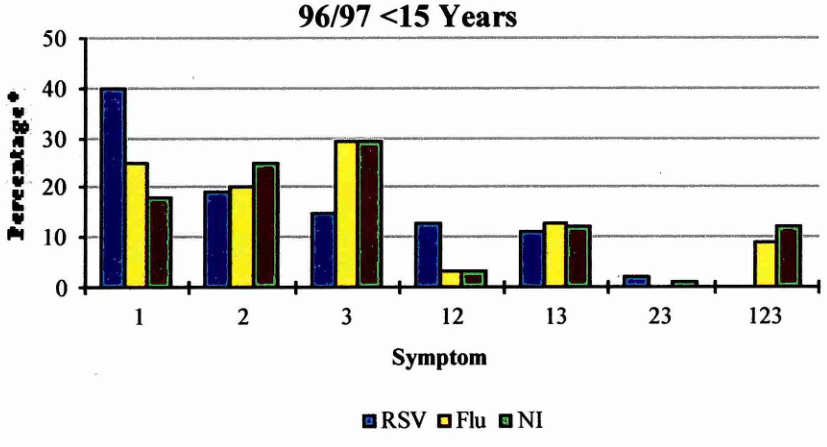
Figure 4.17 Analysis of RCGP results compared with CDSC reports of RSV infection (96/97 winter season)



Laboratory reports to CDSC are of confirmed cases of RSV, mainly from children, from hospitals throughout the UK

Symptoms were recorded in three categories by the GP and reported when the sample was sent to the laboratory for analysis, as for the 95/96 winter season. The analysis of symptoms for the under 15 years old age range is shown in fig 4.18. In this age bracket there were 62 RSV positive samples, 69 influenza positives, and 77 for which no viral pathogen was identified (NI). A large number of the RSV positive patients in the under 15 age range presented with URTI (40%), as with the previous season. Samples with influenza identified as the viral pathogen were marginally more in the fever/constitutional symptom category (29%). However, unlike the previous season the samples for which no viral pathogen was identified were more in the fever constitutional symptom category (29%).

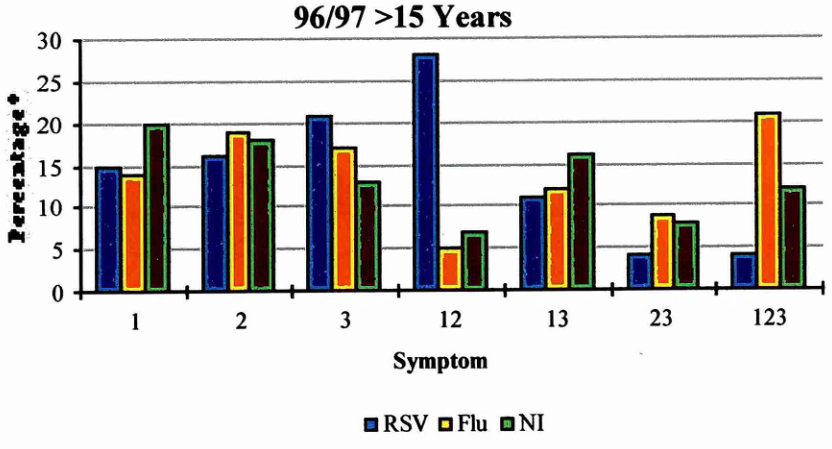
Figure 4.18 Symptoms analysis: Individuals under 15 years old (96/97 winter season)



Key to symptom category's scored by the GP upon collection of the sample
1- URTI 2-LRTI 3-Consitutional
12-URTI & LRTI 13-URTI & Constitutional 23-LRTI & Constitutional
123- URTI & LRTI & Constitutional

Analysis of symptoms for the over 15 years old age range is shown in fig 4.19. For the over 15 age group there were 125 RSV positive samples, 242 influenza positives, and 232 samples for which no pathogen was identified. In comparison with the under 15 years old age range, the majority of RSV positive patients presented with a combination of URTI and LRTI (28%). For influenza many of the patients presented with URTI, or LRTI, or fever, or a combination of all three. The samples for which no viral pathogen was identified came mainly from patients presenting with URTI, or LRTI, or fever, or a combination of all three.

Figure 4.19 Symptom analysis: Individuals over 15 years old (96/97 winter season)



Key to symptom category's scored by the GP upon collection of the sample
1- URTI 2-LRTI 3-Consitutional
12-URTI & LRTI 13-URTI & Constitutional 23-LRTI & Constitutional
123- URTI & LRTI & Constitutional

A total of 32 dual infections were identified during this season, and analysis of these is shown in Table 4.7. Of these 22 (69%) were one influenza subtype associated with a RSV subtype. There were three dual infections identified which involved two influenza subtypes, and seven which involved two RSV subtypes. No infections were identified which involved more than two viruses. Of the dual infections, eight were in children (less than 15 years old) and 24 in adults. The symptoms associated with the dual infections were the same as those associated with single infections; the severity of illness was not recorded.

Table 4.7 Analysis of dual infection reports (96/97 winter season)

Virus Combination	Number of samples
Influenza A H3N2 & RSV A	12
Influenza A H3N2 & RSV B	1
Influenza A H3N2 & Influenza B	3
Influenza B & RSV A	8
Influenza B & RSV B	1
RSV A & RSV B	7
Total	32

The data for the 96/97 season is summarised in Table 4.8. A total of 498 samples (62%) had a viral pathogen identified with the multiplex PCR.

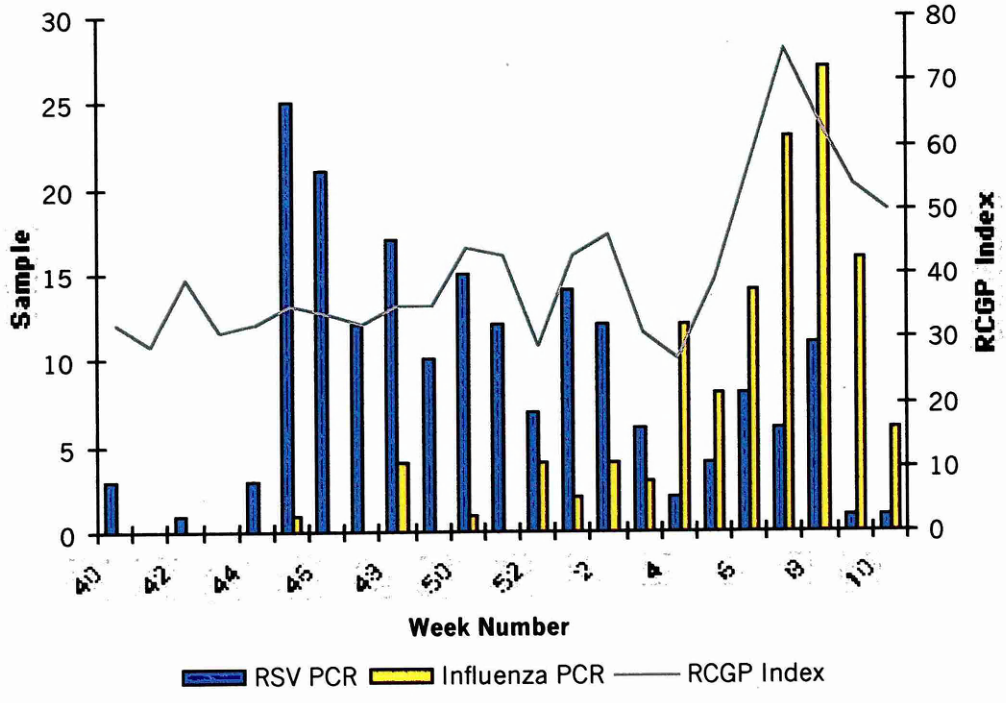
Table 4.8 Summary of data for the 96/97 winter season

	Total	RSV	Influenza	Dual
Number of Samples	807	187	311	32
% (of total)		23	39	4

1997/98 Winter season

A total of 791 combined nose and throat samples were tested. These are the analysis of the results generated by other members of the respiratory virus unit using the asssay described in Chapter 3. A few early RSV infections were identified in weeks 40 and 42, with the RSV season starting in week 44 (fig 4.20). Peak activity for RSV was in week 45, with a decrease in activity around week four before rising again, giving two main peaks of activity. The influenza season did not begin until around week 52, with peak activity in week eight. The rise in influenza activity corresponded with a rise in the RCGP index, unlike the RSV peak activity, which was not reflected in the RCGP index. Influenza activity for this season was uncharacteristically low in comparison with recent years.

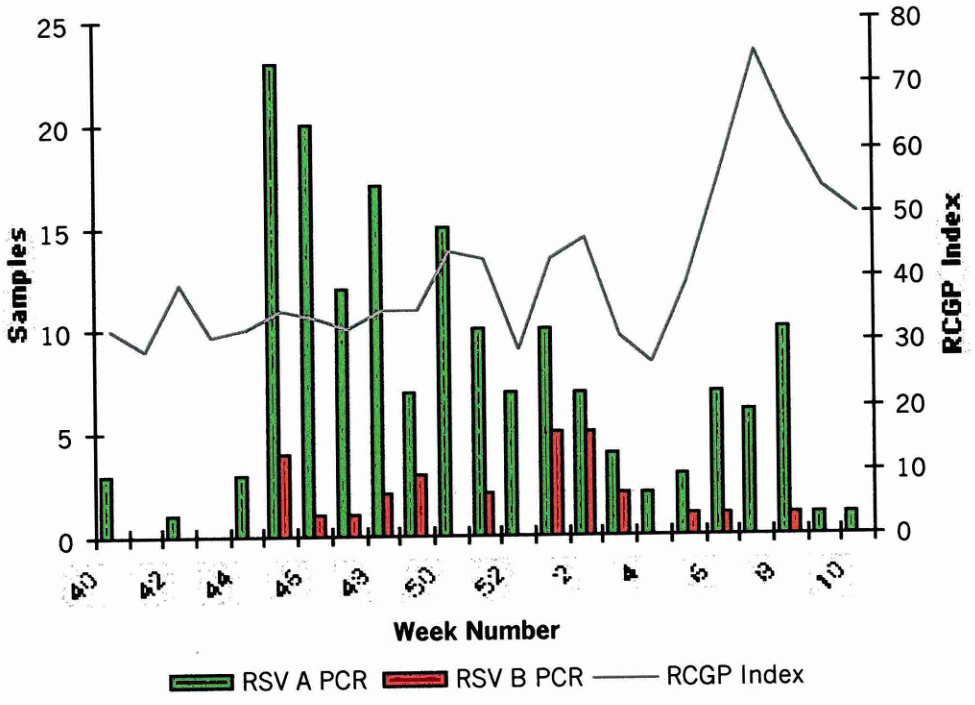
Figure 4.20 Week by week distribution of RSV and influenza as determined by multiplex PCR (97/98 winter season)



The RCGP index represents the consultation rate per 100, 000 of the UK population for influenza or influenza like illness (ILI).

RSV A was the predominant subtype throughout the season with peak activity in week 45 (fig 4.21). Two main peaks of activity were seen with subtype A, which was unlike RSV B, which showed peak activity in weeks one and two. The RSV B season started in week 45 and ended in week eight. RSV A was first seen in week 40 and ended in week 10.

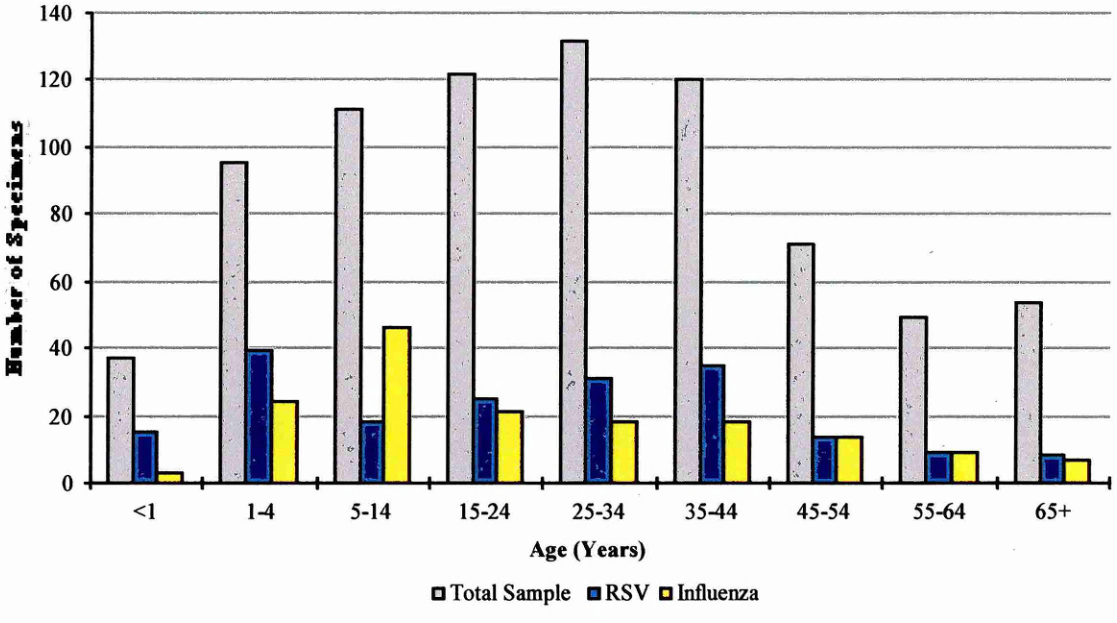
Figure 4.21 Week by week breakdown of the subtypes of RSV (97/98 winter season)



The RCGP index represents the consultation rate per 100, 000 of the UK population for influenza or influenza like illness (ILI).

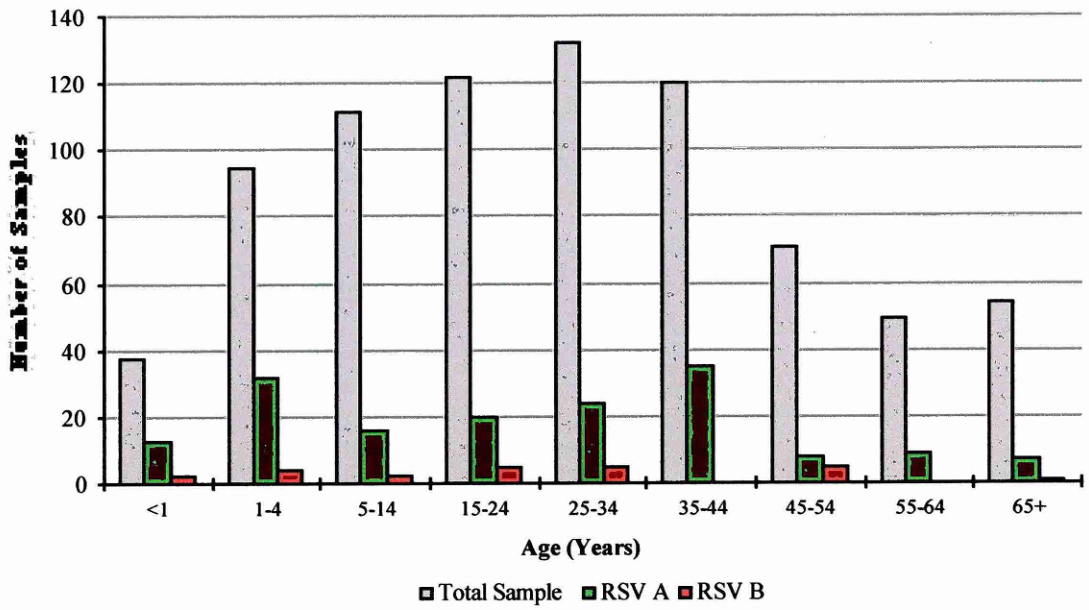
RSV caused disease in all age ranges tested, accounting for more disease than influenza in most age groups (fig 4.22). The age range of the samples was from less than one month to 88 years old, with the mean age of 29 years old. Of the total samples, 31% were from children (less than 15 years old). Only in the age group 5-15 years did influenza account for more respiratory illness than RSV.

Figure 4.22 Age distribution of influenza and RSV infections (97/98 winter season)



RSV A caused infection in all age groups whereas RSV B caused infections in all age groups except, for the older ones (fig 4.23).

Figure 4.23 Analysis of the age breakdown of the RSV positive patients correlated with subtype (97/98 winter season)



The analysis of the total burden of illness of RSV and influenza is shown in Table 4.9. RSV overall accounted for more respiratory infections than did influenza, with the predominant subtype being RSV A.

Table 4.9 Percentage burden of illness caused by RSV and influenza out of the total samples sent (97/98 winter season)

	Total	RSV A	RSV B	Total RSV	Influenza
Number of Samples	791	164	24	194	160
% (of total)		21	3	25	20

The sex distribution of the patients compared with their infections is shown in Table 4.10, and was roughly evenly distributed in every category.

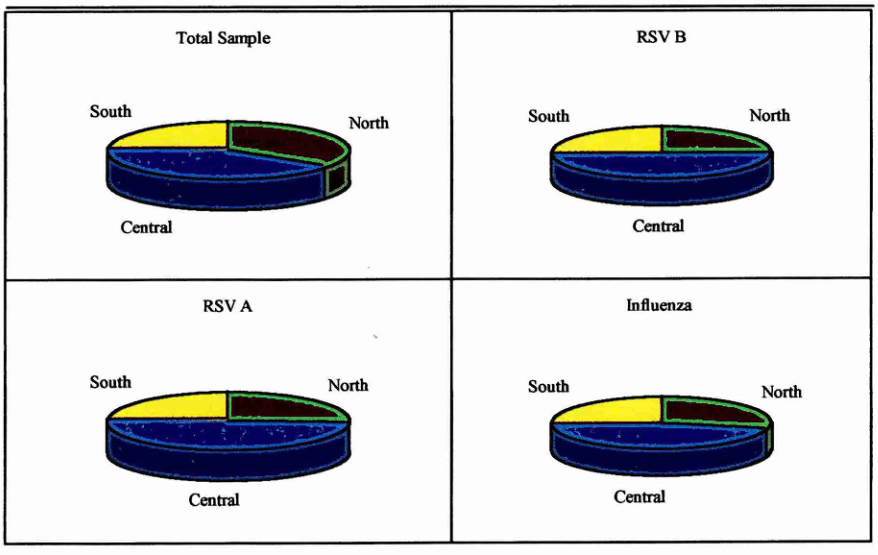
Table 4.10 Sex distribution of RSV infections (97/98 winter season)

Sex	RSV A	RSV B	RSV A+ B	Influenza	Total Specimen
Male	76	13	4	78	346
%*	10	1.6	0.5	10	44
Female	88	11	2	82	445
%*	11	1.4	0.2	10.4	56

* % of total for that group

As for the previous two winter seasons, the majority of samples sent for analysis were from the central region of the country. The distribution of each of the viruses were similar to the total sample distribution (fig 4.24).

Figure 4.24 The geographical spread of numbers of samples correlated with RSV and influenza detection (97/98 winter season)

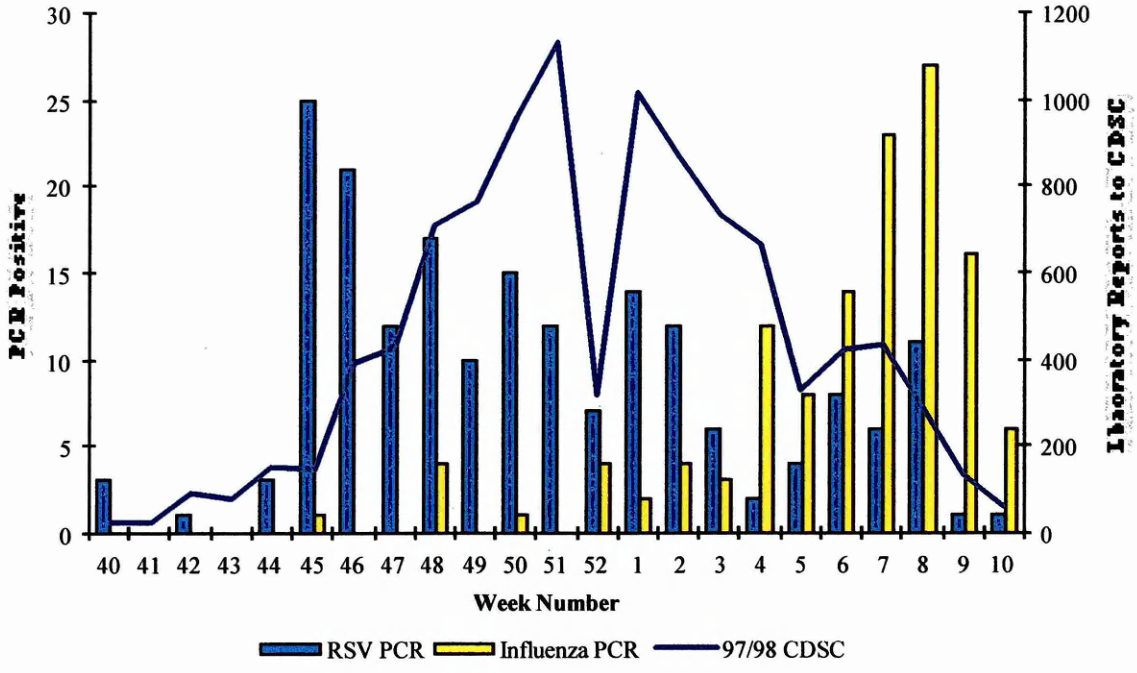


Colour Key

South = yellow, North = green, Central = blue

A comparison of the data from the RCGP and the CDSC reports is shown in fig 4.25. The CDSC reports of RSV peak a little later than the RCGP sample analysis, but both show two main peaks of RSV activity. The CDSC peaks of RSV activity are very close together and are only separated by four weeks, whereas the two peaks of RCGP samples analysis of RSV activity appeared to be separated by 15 weeks.

Figure 4.25 Analysis of RCGP samples compared with CDSC reports of RSV infections (97/98 winter season)

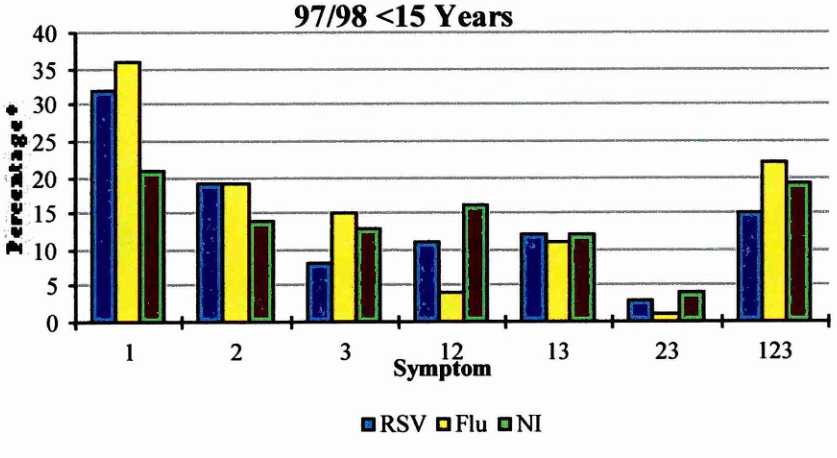


Laboratory reports to CDSC are of confirmed cases of RSV, mainly from children, from hospitals throughout the UK

Of the samples which were RSV positive (n=194), 73 (38%) were from children (under 15 years old). The age range of the patients from whom the samples were taken was from one month to 88 years, with a mean age of 29 years.

Symptoms were recorded by the patient's GP at the time of presentation and sample collection, as for the previous two winter seasons. Analysis of symptoms for the under 15 years old age range is shown in fig 4.26. In this age bracket there were 73 RSV positive samples, 74 influenza positives, and 97 for which no viral pathogen was identified (NI). The majority of the RSV positive patients, in the under 15 age range, presented with URTI (32%). The majority of patients who were influenza positive presented with URTI (36%). The majority of patients who had no viral pathogen identified presented with URTI, or LRTI, or fever, or a combination of all three.

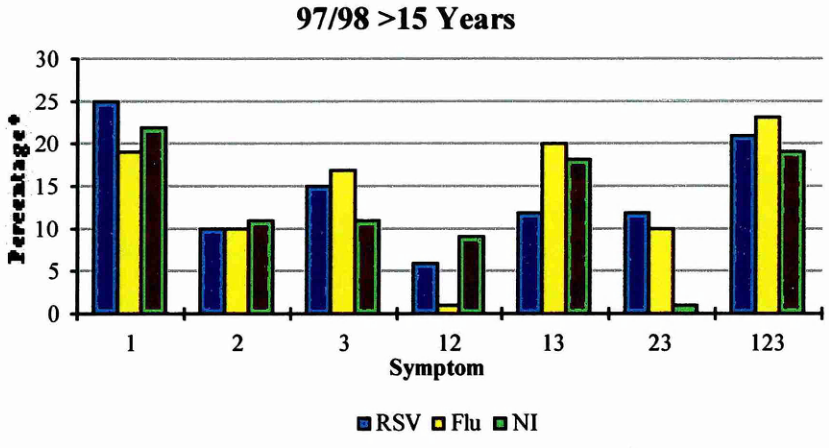
Figure 4.26 Symptom analysis: Individuals under 15 years old (97/98 winter season)



Key to symptom category's scored by the GP upon collection of the sample
1- URTI 2-LRTI 3-Consitutional
12-URTI & LRTI 13-URTI & Constitutional 23-LRTI & Constitutional
123- URTI & LRTI & Constitutional

For the over 15 age group there were 121 RSV positive samples, 86 influenza positives, and 340 samples for which no pathogen was identified (NI). Similar to the under 15 years old age range the majority of RSV positive patients presented with URTI (25%). For influenza positive patients the majority presented with URTI, or LRTI, or fever, or a combination of all three. The majority of patients from which no viral pathogen was identified presented with URTI (22%), unlike the previous two seasons. An analysis of symptoms for the over 15 years old age range is shown in fig 4.27.

Figure 4.27 Symptom analysis: Individuals over 15 years old (97/98 winter season)



Key to symptom category's scored by the GP upon collection of the sample
1- URTI 2-LRTI 3-Consitutional
12-URTI & LRTI 13-URTI & Constitutional 23-LRTI & Constitutional
123- URTI & LRTI & Constitutional

Statistical analysis of the symptoms

Analysis of the symptoms revealed that in the children a significant association with virus recovered could be seen ($P<0.001$). The clearest association was between RSV and URTI, but also presentation of LRTI was more frequently associated with patients from which no virus was identified by multiplex PCR. There was also an excess of fever in those patients from which influenza was detected, however this was less obvious than the other two correlations. The analysis of symptoms in the adult age group (over 15 years old) did not

reveal any associations between the virus isolated (or those patients from which no virus was detected) and symptom presentation. Formal testing, using a log-linear model, of the association between symptom and viral detection in the different age groups gave a likelihood ratio test statistic of 15.73 on six degrees of freedom (P=0.02). Therefore there is evidence that the association is different between adults and children

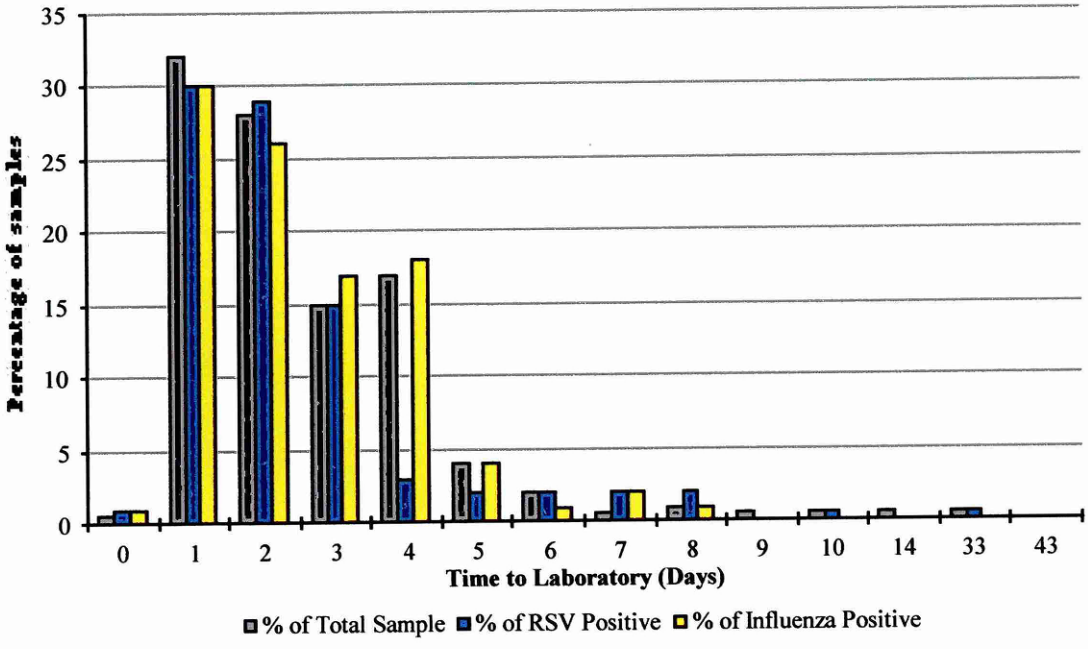
A total of 25 dual infections was identified during the 97/98 winter season, and analysis of these is shown in Table 4.10. Of these 16 (64%) were an influenza subtype and a RSV subtype. There were three dual infections identified which involved two influenza subtypes, and six which involved two RSV subtypes (Table 4.11). No infections were identified which involved more than two viruses. Of the dual infections 12 were in children (less than 15 years old) and 13 in adults. The symptoms associated with the dual infections were the same as those associated with single infections; but the severity of illness was not recorded.

Table 4.11 Analysis of dual infections (97/98 winter season)

Virus Combination	Number of samples
Influenza A H3N2 & RSV A	3
Influenza A H3N2 & RSV B	4
Influenza A H3N2 & Influenza A H1N1	3
Influenza B & RSV A	1
Influenza A H1N1 & RSV A	7
Influenza A H1N1 & RSV B	1
RSV A & RSV B	6
Total	25

The time taken for specimens to arrive for analysis at the laboratory was analysed for the 97/98 season, as the data was complete for this season. The majority of samples arrived within three days from collection of the sample (fig 4.28). Likewise, the majority of specimens from which viral RNA was detected were from samples arriving over this time. Although RSV was detected in samples taking longer than ten days to arrive in the laboratory, the number of samples with detectable RSV RNA fell dramatically when compared with influenza in samples taking four days to arrive for analysis (fig 4.28).

Figure 4.28 Time to laboratory analysis (97/98 winter season)



The data for the 97/98 season is summarised in Table 4.12, a total of 354 samples (45%) had a viral pathogen identified via multiplex PCR.

Table 4.12 Summary of data for the 97/98 winter season.

	Total	RSV	Influenza	Dual
Number of Samples	791	194	160	25
% (of total)		25	20	3

Analysis of the three winter seasons 1995-98

Analysis of the age distribution of all three winter seasons studied is shown in fig 4.29. The data for the 95/96, 96/97 and 97/98 season is summarised in Table 4.13. The weekly analysis of all three winter season studied is shown in fig 4.30.

Figure 4.29 Analysis of age distribution of all samples studied (95-98)

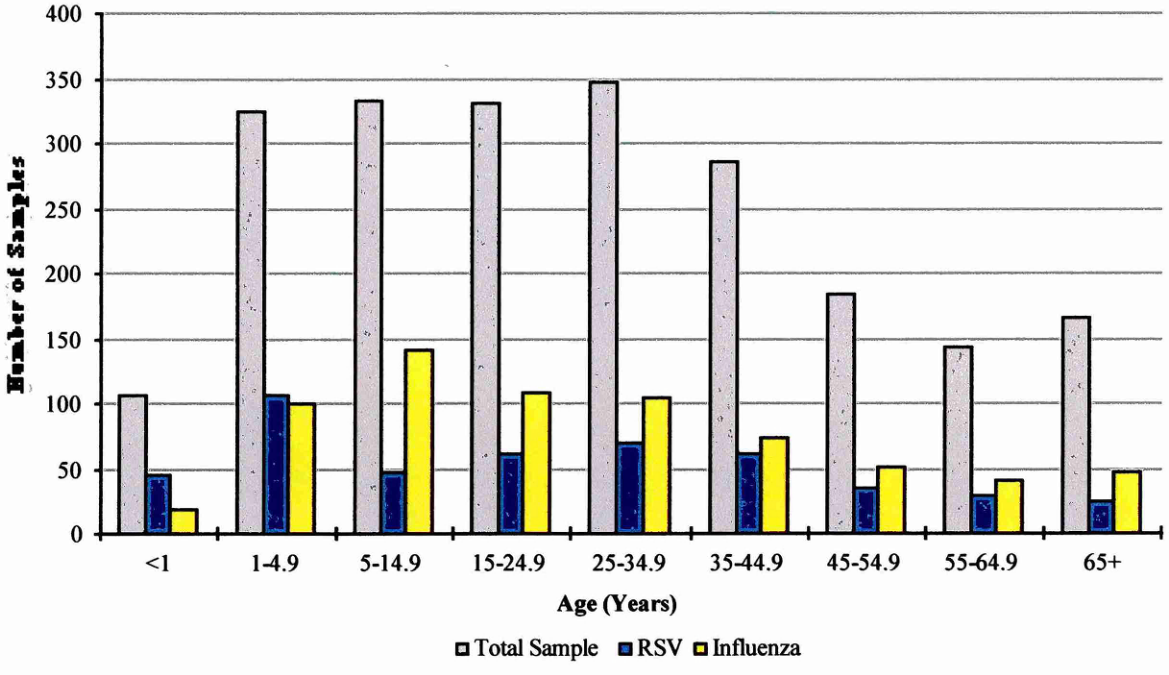
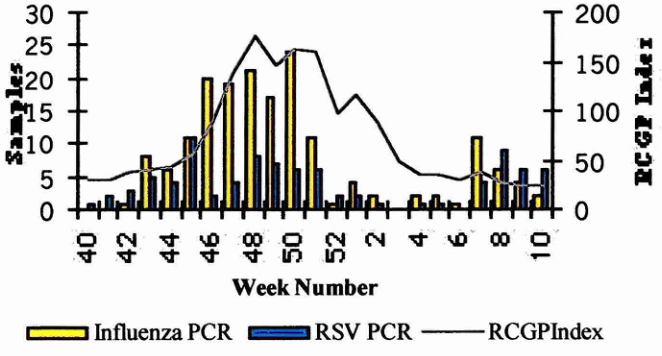


Table 4.13 Summary of all three winter seasons data

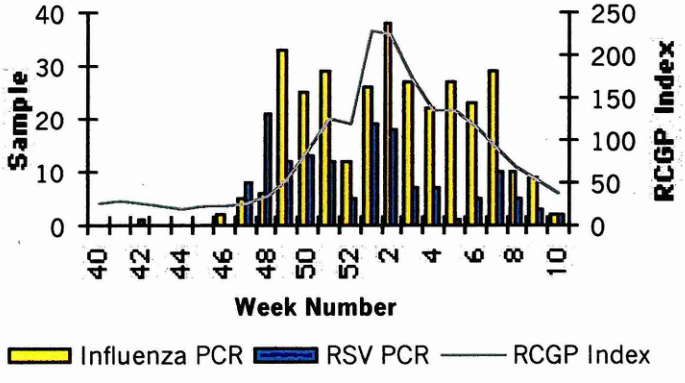
	Total sample	RSV Positive	Influenza Positive	Dual
1995/96	619	97	218	17
1996/97	807	187	311	32
1997/98	791	194	160	25
Final total	2217	478	689	74
%*		22	31	3

* % of final total

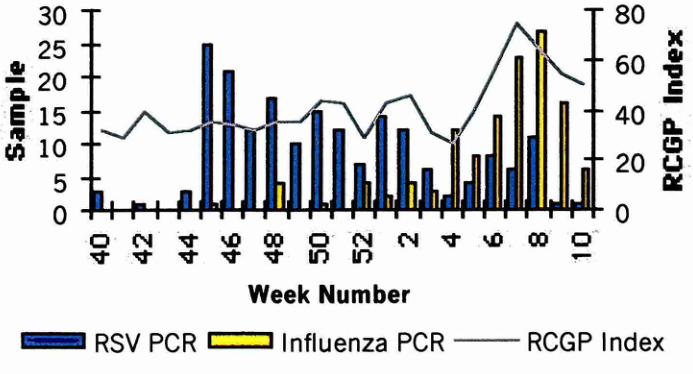
Figure 4.30 Week by week analysis of the RSV and influenza positives for all three winter seasons



95/96 Winter Season



96/97 Winter Season



97/98 Winter Season

The RCGP index represents the consultation rate per 100, 000 of the UK population for influenza or influenza like illness (ILI).

Discussion

RSV was detected in all three winter seasons studied and in all age ranges of the community. For the 96/97 and 97/98 winter seasons the RSV activity was around the same level with roughly 24% of patients having detectable levels of RSV. The RSV activity for the 95/96 winter season was lower, however, at around 16% of patients having detectable levels of RSV. Both subtypes of RSV were found co-circulating with each other and with influenza, although the detection of RSV B was far lower that RSV A for each winter season studied.

Differences between seasons

Samples

There were some differences between the distribution of RSV in the three years studied. The PCR analysis of the 95/96 and 96/97 season was mainly done with stored cDNA, which had already been used once before in separate experiments. Only repeat analyses involved re-extraction from the original specimen. The 97/98 season was extracted from the original specimen.

The use of the stored cDNA may have increased the possibility of sampling bias. On collection the original sample is put in 3 ml of virus transport medium. Upon arrival at the laboratory fungizone was added to the sample. For the 96/97 and 97/98 winter season the sample was vortexed at this stage also. Two thirds of the sample was removed for various culture and ELISA analyses for influenza surveillance before storage at -20°C. For the 96/97 and 97/98 winter season aliquots were also made to prevent repeated freeze/thawing of the original sample. This treatment of the sample may increase the possibility of bias in the individual aliquots, especially if the virus was present at a low level. It is reasonable to assume that the samples from the 95/96 season, which were not vortexed before subsequent aliquoting, are more prone to sampling bias.

The cDNA was stored at -20°C until testing, and was defrosted several times before analysis. As the 95/96 season samples were stored for the longest this may account for the lower positivity rate for RSV for this year. Similarly the detection rate for influenza was lower in my work than in a previous study (70). In an earlier study of the 95/96 season 43% of samples were identified as having influenza (70), whereas only 35% of samples were positive for influenza in mystudy (Table 4.1). Degradation of the samples during storage and freeze thawing may explain the loss in detection, as the methods used were of similar sensitivity. In addition for the 95/96 winter season aliquots were not made of the samples upon receipt into the laboratory, which resulted in the original aliquot of sample being repeatedly defrosted and re-frozen for analysis. For the 96/97 and 97/98 winter seasons samples were aliquoted upon receipt to avoid as far as possible repeated thawing and re-freezing of original sample. This may have also been a contributory factor to the lower detection of RSV during the 95/96 winter season compared with the following two winter seasons studied.

The storage of the samples may not have been optimal for the detection of RNA as they were kept for long periods of time (93). For the detection RNA retrospective testing projects may be improved if the sample aliquots to be tested with molecular methods are put in guanadinium based buffers for storage (93). Of course, it would be best if this type of testing was conducted as soon as the sample arrived in the laboratory, as this would lessen any problems due to degradation of the sample.

Geographical distribution of practices

For all three winter seasons, samples from the central part of the country were unevenly represented. This is particularly pronounced in the first season analysed in this work (95/96). The principal organiser of the RCGP study is based in the central area and, therefore, recruitment from this area may be higher. Over the three years of the study this bias became less apparent, presumably due to the recruitment of more practices around the country. This gave the sample set a more representative geographical spread. The recruitment of more practices would also account for the increase in sample numbers for

the latter two years. The distribution of the sample positives reflected the total sample distribution for every winter season studied.

Communication between the laboratory and the general practices involved with this study improved after the 95/96 winter season. These improvements included better feedback from the laboratory to the practices and, also, more informative instructions on the correct way of taking a combined nose and throat swab. As RSV is tightly cell associated the correct method of taking the swab is essential to recover any of the viral material present. The improvement in detection of RSV between the 95/96 winter season and the two winter seasons studied after it may also be, in part, reflected through these improvements.

RSV A and B co-circulated together with influenza for all three years studied. RSV A was the predominant subtype in each season, whereas the RSV B activity was very low. Previous studies on RSV were contradictory as to which is the predominant subtype (100, 110). Studies which have been conducted over several years have found that the predominant subtype varied between RSV A, RSV B, and equal circulation of both subtypes (100, 110). It has been reported that RSV B does not cause as severe as symptoms as RSV A (241). This may be an explanation for the fewer number of RSV B infections that were detected compared with RSV A. It may be that disease symptoms due to RSV B are simply not as severe as RSV A, and therefore patients will not visit their GP to be diagnosed and sampled. However other studies have not supported the hypothesis that re-infections with RSV are not as severe (234). It is known that RSV is a labile virus, and one possibility is that RSV B does not survive transit as well as RSV A. As only three winter seasons were studied it may be that the level of RSV B would rise as seen in other studies over longer periods of time (100, 110).

Another reason why fewer RSV B infections were detected may be due to the failure of the assay to detect them. This failure could be due to a primer mismatch, however another independent test (an infectivity assay) had a lower positivity for RSV than the multiplex. It would be reasonable to assume that if there was a mismatch between the primers and the template, the positivity rate would be less than the infectivity assay. It remains a possibility,

however, as the optimisation of the multiplex was conducted on laboratory grown material which may have drifted significantly from the circulating strains in the regions amplified. This is unlikely, however, as the regions chosen for amplification are highly conserved, at least in database sequences. Tests on the lability of the individual subtypes would require analysis of samples containing known amounts of either of the subtypes, with a range of conditions. Using an assay which required infectious virus, for example tissue culture, in conjunction with an assay which does not, for example PCR, would also allow a better assessment and comparison of the relative lability of each virus.

During this study dual infections were identified in approximately 3% of patients. For the majority of these dual identifications of one or both of the viruses involved was confirmed by another method of testing such as virus culture or infectivity assay. It is not known to what extent the two viral genomes detected contributed to the disease symptoms of the patients. The multiplex PCR technique is a very sensitive method which may be capable of detecting viral genomes for a period of time after symptoms have abated. However it is not known how long PCR is capable of detecting viral RNA after the initial infection. Experiments to test this would require human volunteer testing with samples taken before, during and after the symptomatic phase of illness. It is not possible to ascertain whether both viruses detected are causing disease, from the present work, as it was done in retrospectively. It is likely, however, that some of the symptoms were due to both viruses. It would be interesting to know if the symptoms associated with dual infections are more severe or longer in duration than those associated with single infections. However, studies of this would require human volunteers. Drews *et al* (1997) noticed that RSV was associated with many dual infections, a finding confirmed by my work where the majority of dual infections were associated with RSV A or B (66).

1997/98 influenza winter season

This season was an unusually mild year for influenza, with reported activity very low during the beginning of the season (5). However this did not seem to affect the RSV season, which followed the same pattern as seen in the previous years. The number of samples sent for analysis that year was similar to that of the year previously, which would suggest that there was another pathogen responsible for the symptoms at the beginning of that season. Whatever affected the influenza virus circulation for the 97/98 winter season showed no apparent effect on RSV circulation.

The time samples took to arrive in the laboratory from the point of collection was analysed for the 97/98 season, as the data was complete (unlike the 95/96 and 96/97 winter seasons). Although in this study the samples were not analysed immediately upon receipt, the time the samples spent in transit to the laboratory may effect the recovery of nucleic acid. This may be of importance, especially for RSV containing samples, as RSV is labile. The majority of samples arrived into the laboratory within three days from collection (fig 4.28). Although few samples took longer than six days to arrive in the laboratory, of the samples which did take longer than six days, some still had detectable viral RNA. This does not mean that the virus would still be infectious and therefore able to be grown. There is a noticeable decrease in the levels of RSV detection in samples taking four days to arrive in the laboratory, compared with influenza (fig 4.28). It is impossible to say whether this is due to the lability of RSV, with the prolonged transit causing degradation of the virus or whether RSV was simply not present in those samples to start with. It is likely, however, that as the recovery rate for influenza and RSV is similar in all other samples sets with respect to time spent in transit, that this does reflect the labile nature of RSV. Also it may be that the virus would be detectable if tested upon receipt in the laboratory, but the combination of prolonged transit, long term storage, and freezing and thawing of the specimen all contribute to reduced detection.

RSV seasonality

The start of the three RSV seasons studied differed slightly. The 95/96 season started early and gradually built up to peak activity in week 45. The last two seasons studied had a more explosive start, with peak activity without a gradual build up. The twin peaks of RSV activity were observed in every year studied, although they were less pronounced in the 96/97 winter season. The question of why RSV activity decreases around the Christmas and the New Year period is difficult to answer. A similar phenomenon can be observed with influenza, but this is usually associated with influenza A subtype decline before the influenza B subtype (or other influenza A subtype) rise. This may be the case with RSV, where one lineage of RSV takes over circulation from another during the winter season. It remains to be seen whether this decrease in detection of virus is a real phenomenon or whether it is artefactual due to the inherent limitations of this type of study. The total number of specimens received into the laboratory falls dramatically around the Christmas and New Year period (on average 40-50%) mainly due to surgery closures. This combined with the lack of a postal service will result in fewer patients being sampled at this time, and may account for the apparent decrease in circulation of viruses.

RSV circulation appears to decrease either for a number of weeks (fig 4.2) or decrease around weeks four to five (fig 4.10 and 4.20) as well as decreasing around week 52. This may reflect the school holidays around this period of the year, often with pupils not returning to school until around week three. This would allow the viruses to spread among the children and then transmit virus to their parents so producing the apparent rise in RSV circulation seen around week seven.

The amount of RSV activity during the summer months cannot be determined through the source of samples used in my work. The RCGP scheme is primarily designed for influenza surveillance, and samples are only collected for analysis during the winter months. Monitoring of data from hospital reports shows very little circulation of RSV during the summer months (personal communication, CSDC). This leaves the question of where the virus goes during the summer months. It may be that RSV, like influenza continually

circulates the globe, for instance peak influenza activity in Australia is during European summer months (101).

Symptoms

No guidelines were given to the individual GPs partaking in the study on what constituted upper, lower or constitutional respiratory symptoms. Therefore, the parameters for analysing these data are broad, however, for the children a significant association between symptom presentation and viral detection was found. For the adults this association was not seen. It would be hard to determine from these data whether the diagnosis of influenza or RSV in children could be affected by clinical presentation, as a clear association between RSV and URTI was observed in children. Further studies would be needed to investigate the association between virus and clinical presentation, with defined criteria for symptom classification.

In this study only severe respiratory illness was studied. In all likelihood, adult patients who experience 'heavy cold' like symptoms would not visit their GP, whereas infants and children who do have these symptoms, would be taken to the GP. These infections may be severe enough for the patient to lose time from work. This would leave a proportion of illness un-sampled and therefore undiagnosed (81). As shown RSV can cause respiratory disease in adults, and it is probable that this number is under representative in this study of the of burden of disease due to RSV.

CDSC reports of compared with RCGP RSV analysis

For the winter seasons analysed, the RCGP index (consultation rate for ILI at General Practitioners throughout the country) mirrored influenza detection, with peak detection of influenza corresponding with peak consultation for ILI (figs. 4.2, 4.11 and 4.20). The RCGP index did not reflect the RSV peak activity as determined by my work (figs. 4.2, 4.11 and 4.20). Comparison of the CDSC hospital reports of RSV for the 95/96 winter season did not reflect the peak activity of RSV as determined by the present work (fig 4.8). The figure obtained for the CDSC reports of RSV infection were from the date the specimen was reported and not the date the sample was received. Although there may be a

delay in reporting of the hospital data to CDSC, the peak activity of RSV, as seen from the CDSC data, was during a period of low RSV activity as seen by analysis of the RCGP samples (fig 4.8). For the following two winter seasons this is not the case and the general pattern of RSV activity is similar between the two data sets (fig 4.17 and 4.25). The CDSC data set is primarily collected from infants and children with severe respiratory illness, whereas the RCGP data set is more representative of the general community. The reasons for the lack of correlation between the RCGP analysis and the CDSC analysis for 95/96 winter season are hard to explain.

Multiplex PCR vs infectivity

The correlation between the multiplex PCR and infectivity assay was poor. The two tests are directed against different targets, and the infectivity assay was done up to one year before the multiplex PCR. The infectivity assay requires live virus whereas the multiplex assay only requires the presence of viral nucleic acid. This may account for the lower sensitivity seen with the infectivity assay as compared with the multiplex PCR assay. Another explanation would be the presence of false positive samples in the PCR testing. The PCR testing was conducted to reduce this possibility to a minimum, with negative controls dispersed throughout the run, to monitor carry-over contamination from neighboring tubes. Also a separate of room for each task (e.g. nucleic acid extraction, PCR etc.) with dedicated equipment and laboratory wear was used. Samples were analysed in size limited runs with known positive controls used at the end of each run.

Therefore, in this study, RSV was found to cause respiratory illness in all age ranges of the general population. For the less than one year olds, RSV was a greater cause of respiratory illness than influenza. For the one to five year olds RSV was almost as a significant cause of respiratory illness as influenza. This would imply that in these age groups GPs are less able to distinguish between the clinical presentation of influenza and RSV. For the five to 15 years old age range RSV only accounted for 8% of the infections identified by the multiplex PCR. For all other age ranges (fig 4.30) RSV accounted for almost half the amount of infections that influenza did. This demonstrates the important role that RSV plays in the burden of respiratory illness in the general community. It also shows that RSV

is a cause of severe respiratory illness in the general adult population. This in turn implies that RSV re-infection, which occurs throughout life, is severe.

Questions arising from this study

The analysis of all the data collected has shown that RSV is an important pathogen causing severe respiratory disease in all age ranges of the community. Studies on the subtypes of RSV isolated from children (22, 191) have shown that the subtypes can be further differentiated into lineages (discussed in Chapter 5).

The questions arising from the analyses previously discussed above include whether, during a RSV season, the same lineage co-circulates in the infant and adult populations? Also are the mixed infections always associated with a particular lineage, or are they representative of those circulating at the time? In addition, is there predominance of a particular lineage which varies annually, as seen in hospitalised children, in the general community?

Chapter 5

Analysis of strain variation in community acquired RSV infections

Introduction

Studies which have analysed the variation between and within the subgroups of RSV have focused both on individual and a combined analyses of RSV genes (22, 88, 191). A strategy for typing strains of RSV has been described using the N, P and SH genes, by restriction enzyme analysis of PCR amplicons (28). More recently research has focused on the G gene of RSV to study strain variation, evolution and immunological response, with the view to creating a vaccine.

Sequence divergence in the G gene

High divergence in the sequence of RSV G is seen between the subtypes of RSV. Divergence of up to 47% has been reported between the two subgroups, with 57% divergence reported in the extracellular portion of the G protein (127). Intrasubgroup variation has been reported to be as much as 20% (26, 225). Over half of the nucleotide changes in the G gene are non synonymous, suggesting that there is a selective pressure for change in this region (225). The cytoplasmic tail and transmembrane portion of the G protein appears to be less divergent, having 84% identity (127).

Mechanisms for change in the RSV G gene

It would seem that there are several mechanisms by which the coding changes in the G gene occur. One of these mechanisms is frameshifting. Here the removal of one base results in a change of reading frames which can completely alter the amino acid produced. It has been noted that this type of frameshifting occurs when there are clusters of adenosines (89, 225). Certain 'hot spots' for the potential for frameshifting in the G with runs of adenosine bases have been identified (24).

The most likely mechanism by which frameshifting events are thought to occur is polymerase error, due to a form of slippage. Frameshifting has been shown *in vitro* to enable the virus to escape neutralisation by monoclonal antibodies (24). Although this was not supported by the study performed by Sullender *et al* (1991), the sample set tested was extremely limited in number, and more extensive analysis of these types of changes would be required before drawing any possible conclusions as the extent of their importance in

allowing reinfection *in vivo*. Another mechanism which changes the amino acid sequence of the G protein involves alternative termination codons at the end of the gene, giving rise to different truncated forms of G proteins (225).

Strain variation

Subgroup A of RSV has been genotyped into six different lineages based on restriction fragment length polymorphism (RFLP) analysis of the SH gene (22). It has also been further broken down into 22 'clades' (191). Subgroup B has been divided into two different lineages on the basis of RFLP on the NP genes (27), and into 6 lineages by analysis of the G gene (191).

Variation in the strains of RSV circulating between 1988 and 1993 and isolated from children in the Birmingham area, were analysed using restriction enzyme digestion of PCR products of the N and SH genes and by sequencing of the G gene (22). A pattern emerged in which the predominant circulating strain varied yearly. The periods in between epidemic predominance for a particular strain saw low to medium activity, or sometimes no activity at all (22). Comparison of these results with others obtained from RSV isolates from Uruguay showed a similarity between the lineages (22). Often more than one genotype was co-circulating in this population during an epidemic. This was supported by another study which analysed RSV isolates from infants in Liverpool by restriction enzyme digestion of N and G amplicons (82). Different lineages were found to co-circulate. However, the pattern of predominance of one particular genotype was only seen in one of the epidemic season covered.

This pattern of co-circulation of different genotypes was also seen in studies conducted on RSV isolates obtained from children over several American States (2). Monoclonal antibodies were used to distinguish between the strains and two epidemic seasons were studied (2). In contrast to the findings of Cane *et al* (1994), differences in the predominant subgroup and strain type (A or B) were seen between the different States in the same epidemic, suggesting that the same predominant strains were not circulating throughout the country at the same time (2, 22).

A study by Johansen *et al* (1997) on isolates from hospitalised children in Denmark used restriction enzyme analysis of F and G gene amplicons. Three epidemic seasons were studied and results showed there to be a general pattern of predominance in the circulating isolates, as found in other studies (22, 125). Further studies of RSV in children over a five year period in the United States also saw a pattern of predominance of certain strains with no one strain predominating more than once in the five years analysed (191). It was speculated from this work that variation in the dominant strain was due to the novel strain being better able to evade immune capture and was therefore circulating more efficiently, or being more pathogenic (191).

Severity of disease associated with individual genotypes

The severity of disease associated with the subtypes of RSV has been discussed in chapter one. Due to the large amount of variation seen within each subtype it is possible that disease caused by certain strains may be more severe than others. Fletcher *et al* 1997 used restriction fragment length polymorphism to assign genotypes to RSV strains isolated from infants with acute respiratory tract infections (82). The severity of the disease was scored retrospectively on clinical presentation of symptoms and the treatment required. This study showed variation in the severity of disease, with one RSV genotype (SHL2) showing more moderate to severe disease than the other genotypes.

In another study into this phenomenon by Sullender *et al* (1998) two pairs of RSV A isolates which caused sequential infections in children were found to differ substantially in their RSV G sequence (226). However, in animal tests it was demonstrated that vaccination with one isolate G protein provided protection against infection with either of the isolates. These results suggest that re-infection occurred for reasons other than variation in strain type through the G protein (226).

Viral quasispecies

Mistakes in replication of RNA genomes cannot be corrected due the lack of proof reading ability of the RNA dependent RNA polymerase. This in turn leads to errors in the progeny RNA genomes. A population of viruses will contain many different mutations and will form a quasispecies. The rate of mutation for influenza virus NS gene has been estimated to be 1.5×10^{-5} (188) and the L gene of VSV mutation rate has been estimated at 10^{-4} (214).

In studies of RNA viruses by DNA sequencing to estimate their diversity there are inherent complications due to enzyme induced errors. Chadwick *et al* 1998 compared three RNA amplification methods used for DNA sequencing (35). The conclusions from their work showed that use of MMLV reverse transcription followed by a nested PCR with *Taq* polymerase gave the least errors, compared with either a combined RT-PCR using a *rTth* enzyme, or a nucleic acid sequence based amplification (NASBA) method with AMV reverse transcriptase and T7 RNA polymerase (35). Another study by Bracho *et al* (1998) focused on the errors introduced by *Taq* polymerase during amplification (16). Here it was concluded that in work where the sequence variation in the samples was low, the choice of enzyme used to amplify the DNA was critical, with a proof reading enzyme such as *Pfu* giving rise to the least errors (16). With an RNA virus the most inefficient step in sequencing will be the reverse transcription stage to produce cDNA. Most studies which have focused on examining strain variation, rather than the quasispecies directly, have not used cloning or proof reading enzymes during amplification. This is acceptable because these comparisons are between consensus sequences, so the same error prone methods can be used. The region amplified may be highly variable (i.e. the G gene) and it can be assumed that the error rate will be consistent between the different templates. The majority species present in these samples is therefore considered as the strain type.

Earlier, RSV as a significant cause of respiratory illness in all ages of the general community was discussed (Chapter 4). An important question arising from this discussion is 'Are the same strains circulating within the general community as within the hospitalised infant population?' Presumably there is transmission from one population group to the other, but it is relevant to ask if certain strains are associated more with the older age ranges

than with the younger? It would be reasonable to assume that the same strains are circulating within the general community as within the infant population. However, this is unknown and the answer to it may have implications for any vaccine development.

Aim

This part of the project was aimed at designing a sensitive PCR amplification and sequencing method for the analysis of the RSV G gene. This was to study strain variation of RSV, and it had to be capable of amplifying RNA from low copy number, poor quality specimens. The assay developed would be used to analyse the RSV positive specimens identified from the three winter seasons. This PCR had to be able to amplify both RSV A and B.

Hypotheses tested

Do the same strains circulate within the adult population as within the infant and child population? Are the strains associated with dual infections the ones that are predominant in the community at that time? Is the same pattern of yearly dominant strain variation seen within the general community as within the hospitalised infant population?

Results

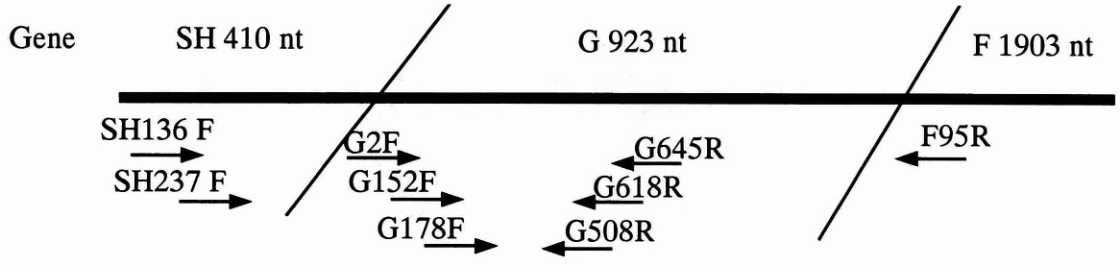
Primers were designed to amplify the first variable region of the G gene (Table 5.1, fig 5.1). These primers were selected to provide the maximum specificity of detection for both subtypes of RSV.

Table 5.1 Primers designed to amplify RSV G gene

Primer	Sequence	Gene	Nucleotide Position*	GC Content %
G2F	gggcaaatgcaaacatgtccaaa	G	4644	43
G152F	ttgcaatgataatctcaac	G	4794	32
G178F	ataattgcagccatcatattc	G	4820	33
G508R	aagttgaacacttcaaa	G	5150	29
G618R	tttgtgggcttggtggtg	G	5260	56
G645R	ttttttggttgtcttg	G	5287	31
G622R	tgttktttgtgggcttggtggtg	G	5264	48/52
F95R	taaaattcttcagtgat	F	5712	24
SH237F	attccataacaaaacctt	SH	4425	28
SH136F	catccataacaatagaattc	SH	4324	30

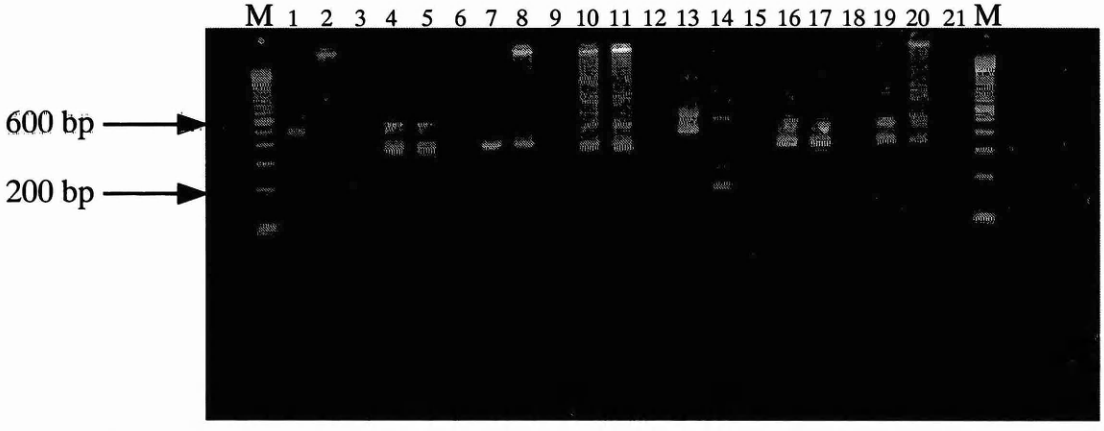
* Ref strain HRU39662 genebank accession No. U39662

Figure 5.1 Diagrammatic representation of primer positions designed to amplify part of the RSV G gene



Testing of the primers, in various combinations, on laboratory adapted strains of RSV was performed and the results are illustrated in fig 5.2. There were several non-specific amplicons synthesised as can be seen, for instance, in lane 5 of fig 5.2 in which unexpected bands are visible. Some reactions were unsuccessful with no amplification occurring, as can be seen, for instance, in lane 2 of fig 5.2, which is lacking the expected band of 380 bp.

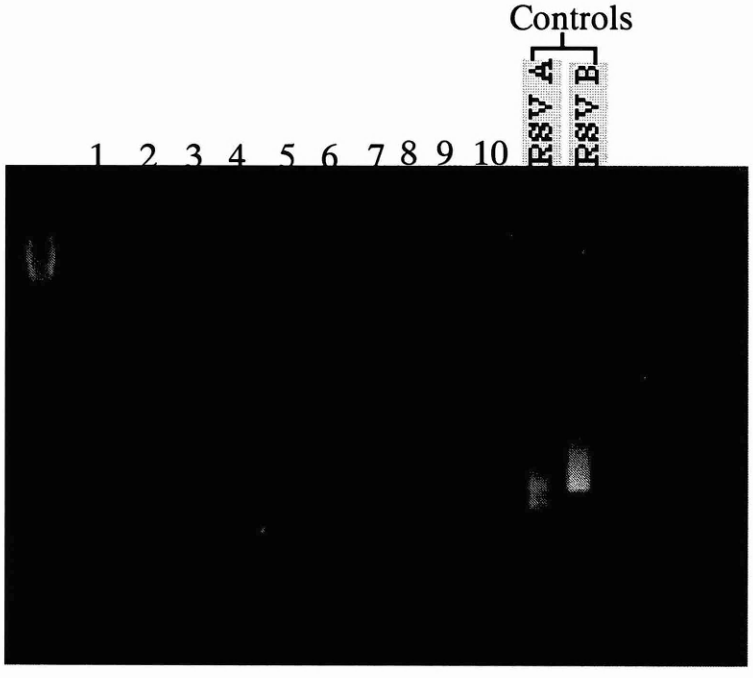
Figure 5.2 Testing of RSV G primers



M is a mobility marker with base pair sizes indicated with arrows. All template material was laboratory adapted strains of RSV. Lane 1 contains RSV A amplified with primers G2F and G645R in the primary, G152F and G618R in the secondary. Lane 2 contains RSV B amplified with primers G2F and G645R in the primary, G152F and G618R in the secondary. Lane 4 contains RSV A amplified with primers G2F and G645R in the primary, and G178F and G508R in the secondary. Lane 5 contains RSV B amplified with primers G2F and G645R in the primary, and G178F and G508R in the secondary. Lane 7 contains RSV A amplified with primers G2F and G618R in the primary, primers G152F and G508R in the secondary. Lane 8 contains RSV B amplified with primers G2F and G618R in the primary, primers G152F and G508R in the secondary. Lane 10 contains RSV A amplified with primers G2F and G618R in the primary, primers G178F and G508R in the secondary. Lane 11 contains RSV B amplified with primers G2F and G618R in the primary, primers G178F and G508R in the secondary. Lane 13 contains RSV A amplified with primers G152F and G645R in the primary, primers G178F and G618R in the secondary. Lane 14 contains RSV B amplified with primers G152F and G645R in the primary, primers G178F and G618R in the secondary. Lane 16 contains RSV A amplified with primers G152F and G645R in the primary, primers G178F and G508R in the secondary. Lane 17 contains RSV B amplified with primers G152F and G645R in the primary, primers G178F and G508R in the secondary. Lane 19 contains RSV A amplified with primers G152F and G618R in the primary, primers G178F and G508R in the secondary. Lane 20 contains RSV B amplified with primers G152F and G618R in the primary, primers G178F and G508R in the secondary. Lanes 3, 6, 9, 12, 15, 18, 21 are negative controls.

Primers previously published (191) were tested on laboratory adapted strains of RSV and clinical material (fig 5.2). Amplification was only seen with laboratory adapted strains at high titres. No amplification was seen with the clinical material as illustrated in fig 5.3, lane 3, in which no amplicon is visible.

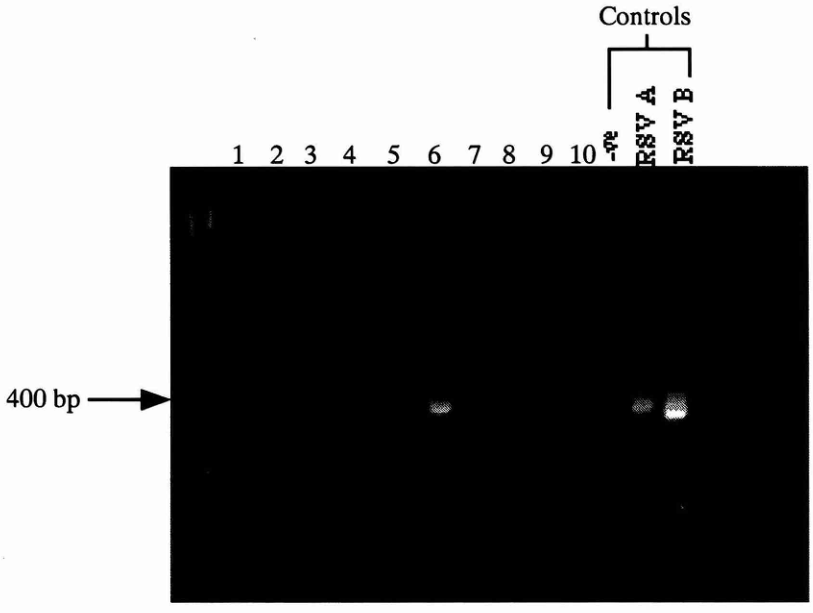
Figure 5.3 Testing of published primers to amplify part of the RSV G gene (191)



M is a marker with base pair sizes indicated with arrows. lanes 1 to 10 contain clinical material amplified with primers described by Peret *et al* (191)

Also tested were primers designed at CDC (during a visit to CDC, Atlanta). The sequence for GABSHORT was unavailable (fig 5.4), and amplification from only one clinical specimen was successful (fig 5.4, lane 6).

Figure 5.4 Testing of the Peret unpublished primers



M is a marker with base pair sizes indicated with arrows. lanes 1 to 10 contain clinical material amplified with primers whose sequence was unavailable.

The primer combinations which were found to give the best amplification and sequencing results are listed in Table 2.

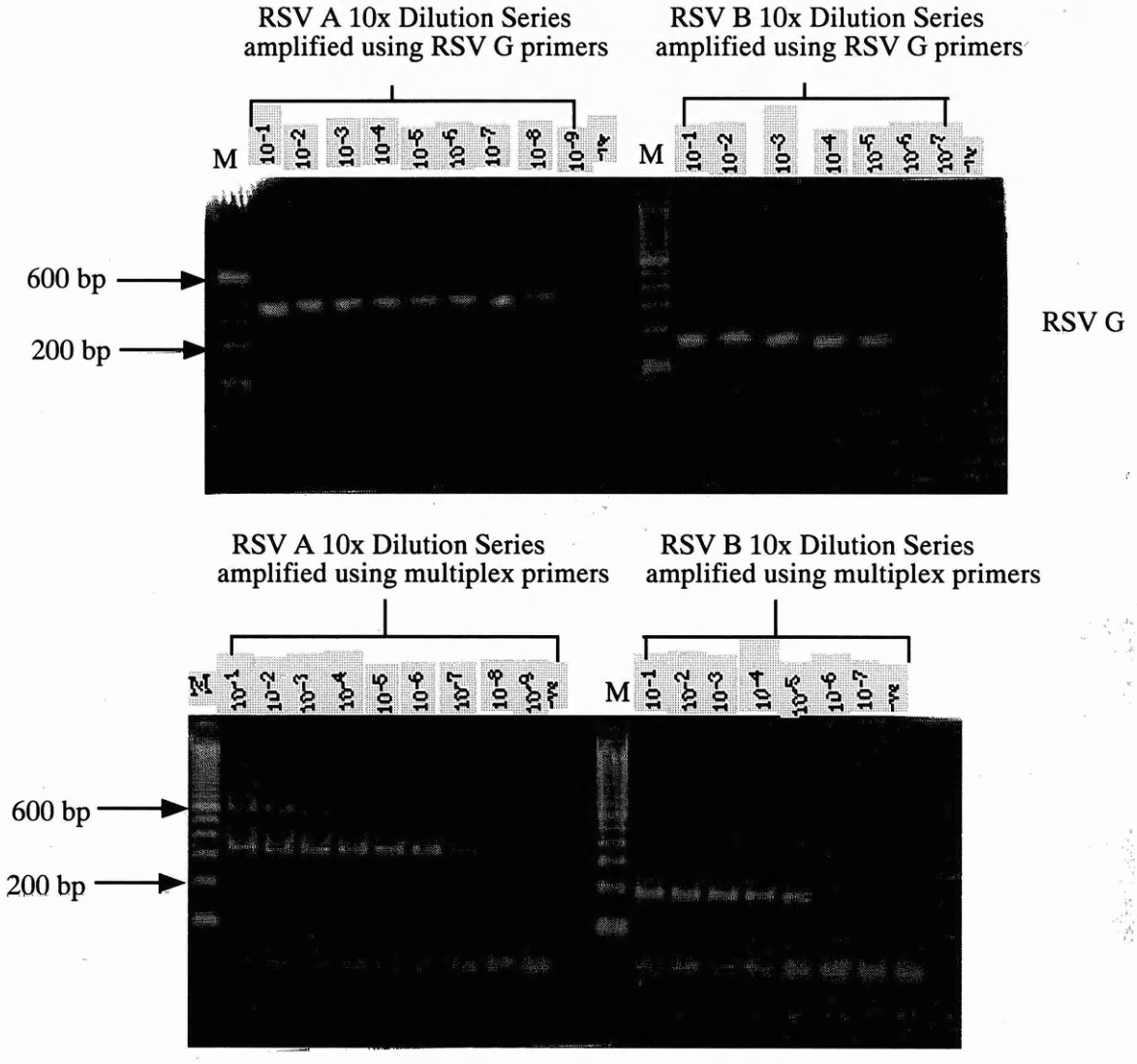
Table 5.2 Final RSV G primer choice

Primary Amplification				
Primer	Sequence 5' → 3'	Gene	Position*	Product size (bp)
G2F	gggcaaatgcaaacatgtccaaa	G	4644	1016
F95R	taaaattcttcagtgat	F	5712	
Secondary Amplification				
G152F	ttgcaatgataatctcaac	G	4794	356
G508R	aagttgaacacttcaaa	G	5710	

* Ref strain HRU39662 genebank accession No. U39662

The DNA sequences of the amplicons made with the primers were compared with database sequences to verify that the correct product was made. Stored clinical samples were used for these experiments. Comparison with the multiplex PCR (Chapter 3) was performed on a 10x dilution series of laboratory adapted material (fig 5.5). Equivalent sensitivities were seen with the two PCR methods on laboratory grown material.

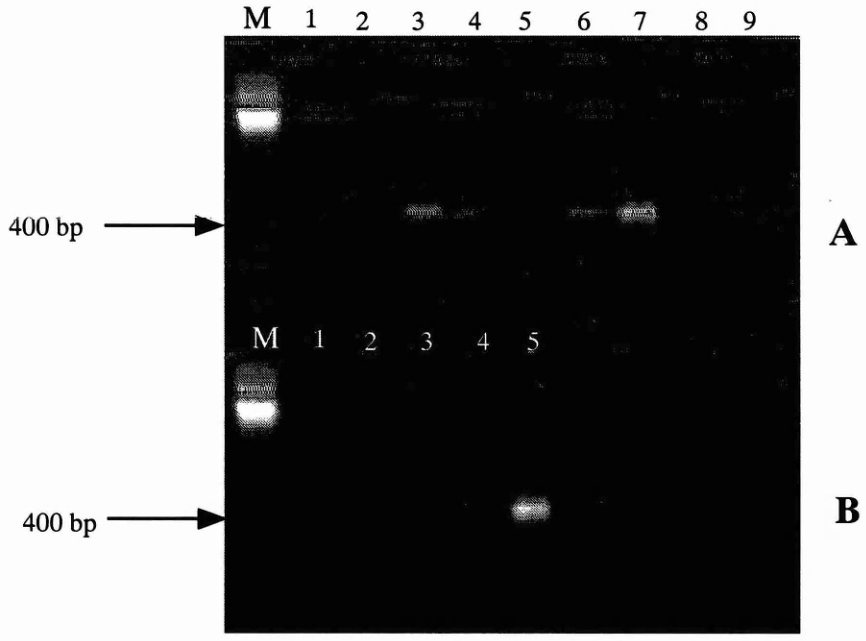
Figure 5.5 Comparison of multiplex and RSV G amplification on RSV A and B templates



M is a marker with base pair sizes indicated with arrows

The RNA levels in the specimens were tested with ß-actin housekeeping primers to assess the extent of RNA degradation. RNA of housekeeping genes should normally be present in abundance. (245) (fig 5.6). Of the 10 clinical samples tested, 8 had detectable levels of ß-actin RNA.

Figure 5.6 ß-actin testing of samples



M is a marker with base pair sizes indicated with arrows. Panel A lanes 1 to 9 contain clinical samples. Panel B lane 1 contains clinical material, lane 2 is a negative control, lane 3 contains RSV A (laboratory strain), lane 4 contains RSV B (laboratory material) and lane 5 contains cellular control material.

The performance of the RSV G sequencing PCR was investigated with aliquots from 10 clinical samples identified as RSV positive by the multiplex PCR from the 97/98 season. One aliquot was extracted and amplified with either the RSV G primer sets or with the multiplex primer sets (fig 5.7). The same degree of amplification was seen with the multiplex PCR as with the RSV G sequencing PCR (fig 5.7). The other aliquot was innoculated onto Hep-2 cells and cultured for one week, with constant monitoring for CPE. The cells were blind passaged twice before being sampled for testing by RSV G sequencing PCR. No more virus could be detected than the initial tests indicated were present.

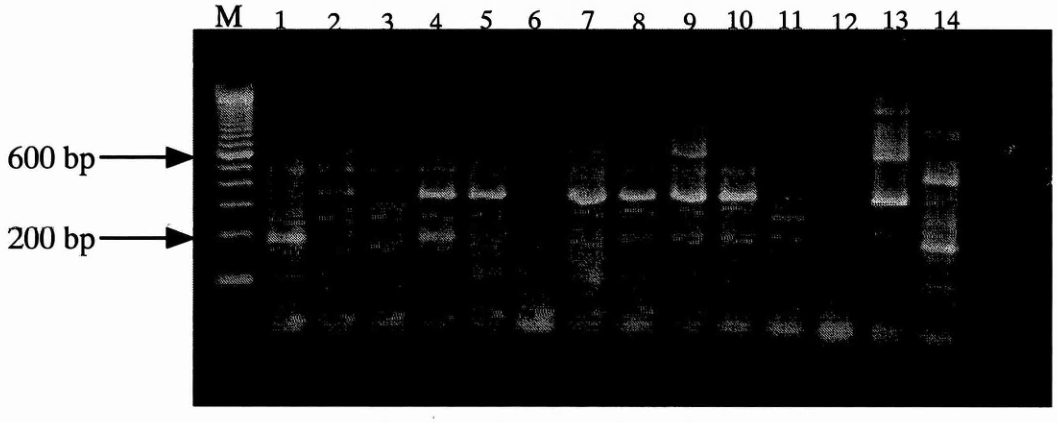
Figure 5.7 Comparison of multiplex PCR and the RSV G sequencing PCR with the same clinical specimens

M is a marker with base pair sizes indicated by arrows. Panel A samples were amplified using multiplex PCR conditions (chapter 3). Lanes 1 to 5, 7 to 11 and 14 are clinical samples. Lane 13 contains RSV A laboratory adapted material, lane 15 contains influenza A (H1N1, H3N2) and influenza B, lane 16 contains RSV A and B laboratory adapted material. Lanes 6 and 12 are negative controls. Panel B samples were amplified using the RSV G sequencing PCR. Lanes 1 to 5, 7 to 11 contain clinical samples. Lane 13 contains RSV A laboratory adapted material, lane 14 contains influenza A (H1N1, H3N2) and influenza B, lane 15 contains RSV A and B laboratory adapted material. Lanes 6 and 12 are negative controls.

Laboratory models showed that the multiplex PCR was capable of detecting RSV in the same samples as did the RSV G sequencing PCR. Tissue culture growth of the samples was conducted for the 97/98 RSV positive samples (as originally identified by the multiplex PCR) and did not yield any further virus positives than those originally identified by the initial RSV G analysis.

Analysis of the 95/96 and 96/97 seasons using the multiplex PCR was completed using stored cDNA samples. For the 97/98 season RNA was extracted from the sample and cDNA was also available for testing. Analysis of some of these stored cDNA samples was conducted to ascertain the longevity of the cDNA. However, only enough cDNA was available for testing by one method, so a comparison between the multiplex PCR and the RSV G sequencing PCR could not be performed. Multiplex PCR on the stored cDNA showed that some degradation could be identified as a smear on the gel, as illustrated in fig 5.8, lane 2. Overall the positivity rate was 70% (fig 5.8). Only three of these samples yielded product with the RSV G amplification from the original sample, by tissue culture and subsequent PCR.

Figure 5.8 Analysis of stored cDNA using the multiplex PCR



M is a marker with base pair sizes indicated with arrows. Lanes 1 to 5, 7 to 11 are clinical samples. Lane 13 contains RSV A laboratory adapted material, lane 14 contains RSV B laboratory adapted material. Lanes 6 and 12 are negative controls.

The 95/96 and 96/97 seasons RSV positive samples (as originally identified by the multiplex PCR) were amplified with the RSV G primers (Table 5.2) and resulting amplicon sequenced. Overall the positivity rate for each season is shown in Table 5.3.

Table 5.3 RSV G PCR sequencing analysis

Season	Total RSV Amplified	Total RSV Sequenced (%*)	RSV A Amplified	RSV A Sequenced (%*)	RSV B Amplified	RSV B Sequenced (%*)
95/96	34	28 (82)	15♦	12 (80)	19	16 (84)
96/97†	34	18 (53)	33	18 (55)	1	0 (0)
97/98	45	17 (38)	37♠	15 (41)	8	2 (25)
Total	113	63 (56)	85	45 (53)	28	18 (64)

KEY

† 152 samples recovered for analysis out of 187

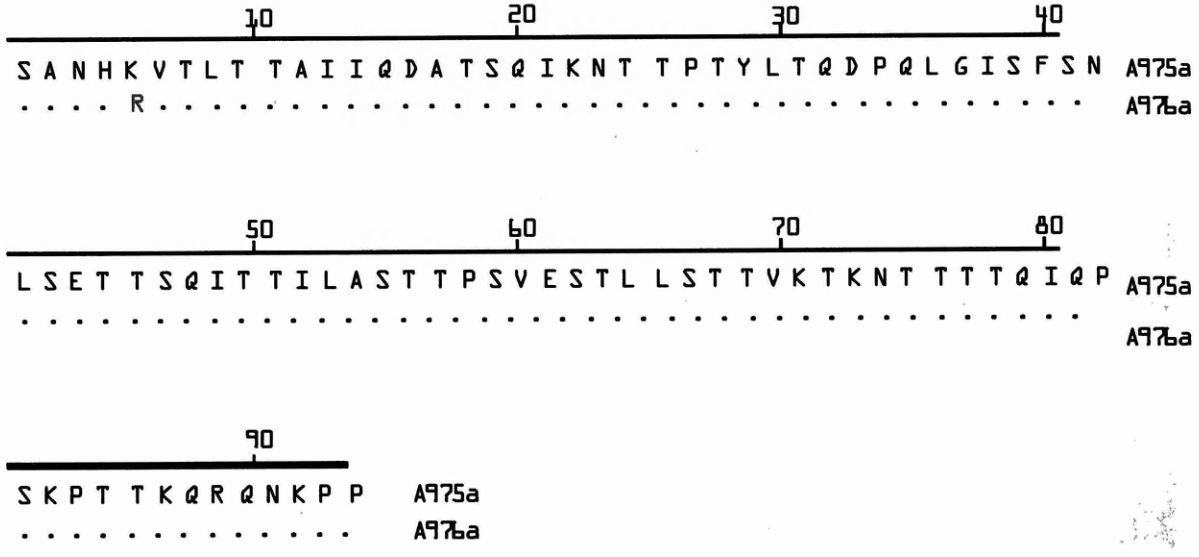
♦ 1 Dual RSV A & B

♠ 2 Dual RSV A & B

* Percentage of total amplified

The sequence obtained directly from the clinical sample was compared with that obtained from the same sample cultured in a Hep-2 cell line for three weeks and passaged twice during this time. This comparison was performed for 5 samples and only one sample showed an amino acid change (fig 5.9).

Figure 5.9 Comparison of amino acid alignments from the same isolate obtained either directly from the original sample or from tissue culture.



Phylogenetic analysis

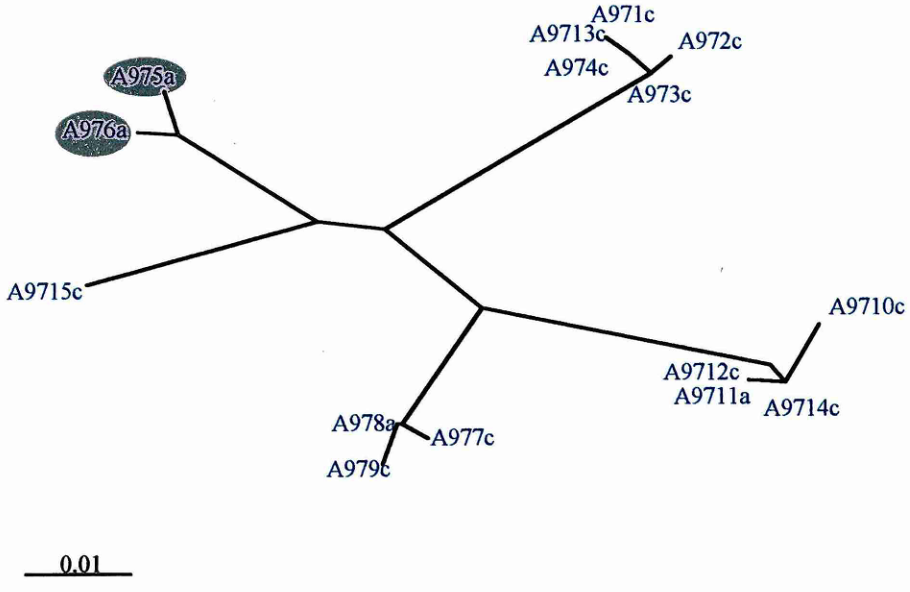
Unrooted trees were created by the distance methods of neighbor joining and maximum likelihood. The maximum parsimony method was not used in this work as it is best suited for sequences which are closely related (43).

i) Neighbor joining: the program Puzzle was used to calculate transition/transversion (Ts/Tv) ratios which were used as a setting in the program DNAdist. The maximum likihood distance option in DNAdist was chosen to give the most robust results. The DNAdist output distance matrices were used in the programs Fitch or Neighbor to create neighbor-joining trees. The significance of these trees was assessed by bootstrapping using the programs Seqboot and Consense.

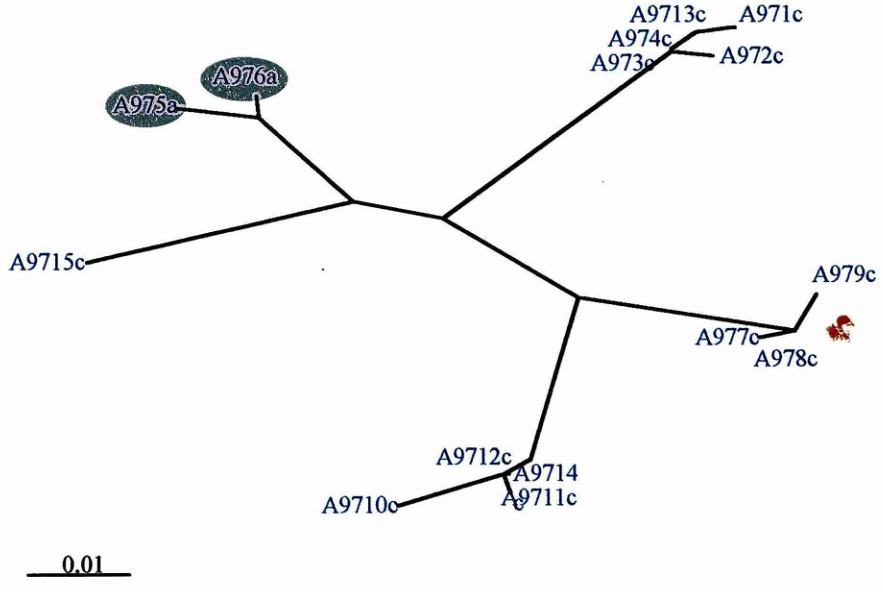
ii) Maximum likelihood: most datasets were analysed by the maximum likelihood method with the program DNAml, using the global rearrangement and jumble options to create the most robust tree. However, bootstrapping from this method is impractical, due to the large amount of computing power required. The maximum likelihood trees were compared with the bootstrapped trees obtained by neighbor-joining (calculated using DNAdist and Neighbor or Fitch). The trees obtained by these two methods were congruent (fig 5.10).

Figure 5.10 An example of two trees produced by maximum likelihood and neighbor joining.

97/98RSV A **A Neighbor joining tree**



97/98RSV A **A Maximum likelihood tree**



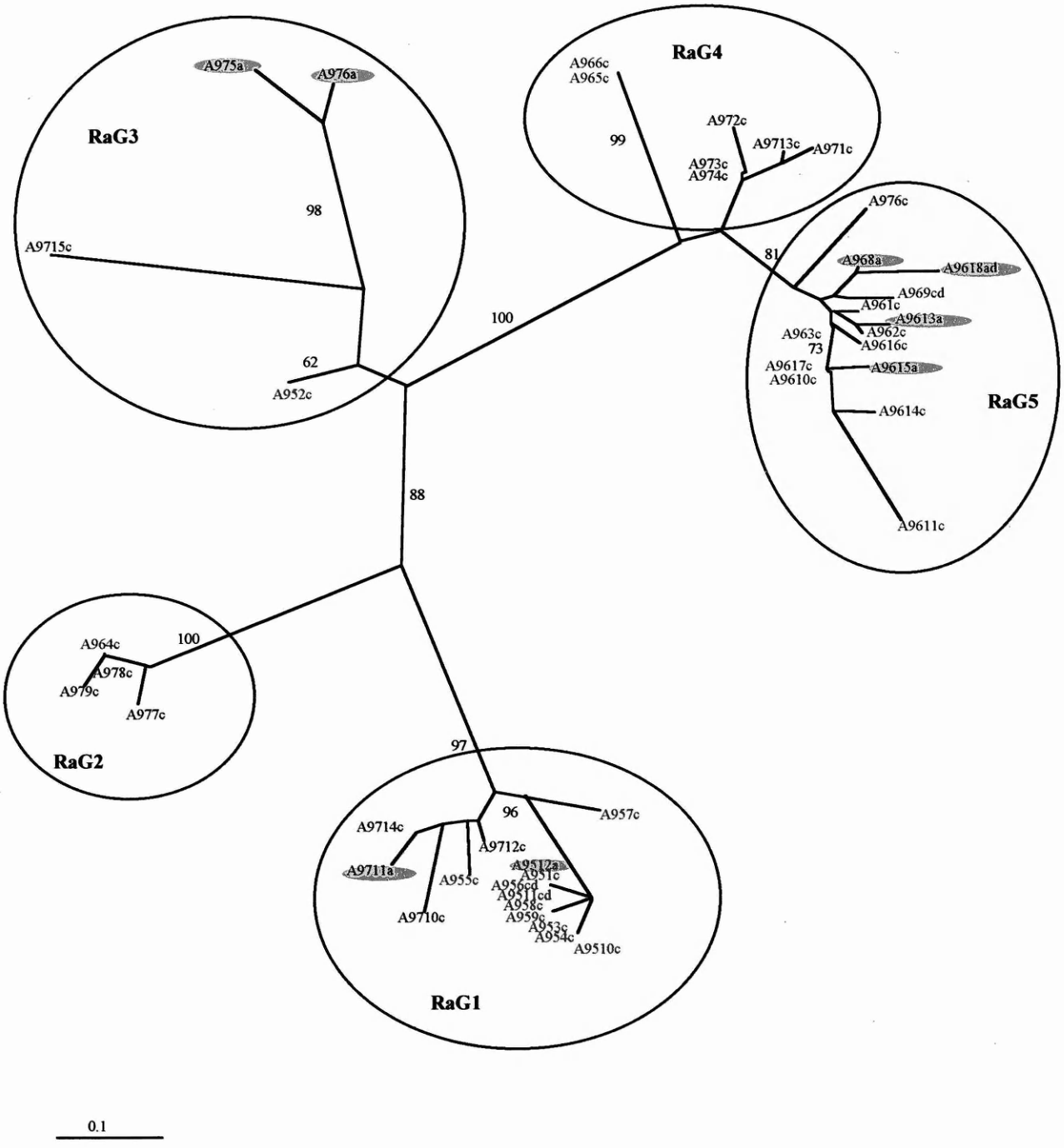
The isolates obtained from adults are shown circled in grey.

Sequencing

Sequencing the RSV G amplicons and phylogenetic analyses of the sequences obtained were done for each season. The RSV A and B subtypes were analysed separately. Strain types were assigned by grouping. All winter seasons were analysed together (figs 5.11 and 5.12) and separately where numbers permitted (figs 5.13, 5.14, 5.15 and 5.16). For every season the two subtypes of RSV co-circulated together with influenza, although the RSV B activity was significantly less than RSV A. Also, different strain types co-circulated with one another (fig 5.11, 5.12), with predominance of one subtype pronounced in the 95/96 and 96/97 winter seasons.

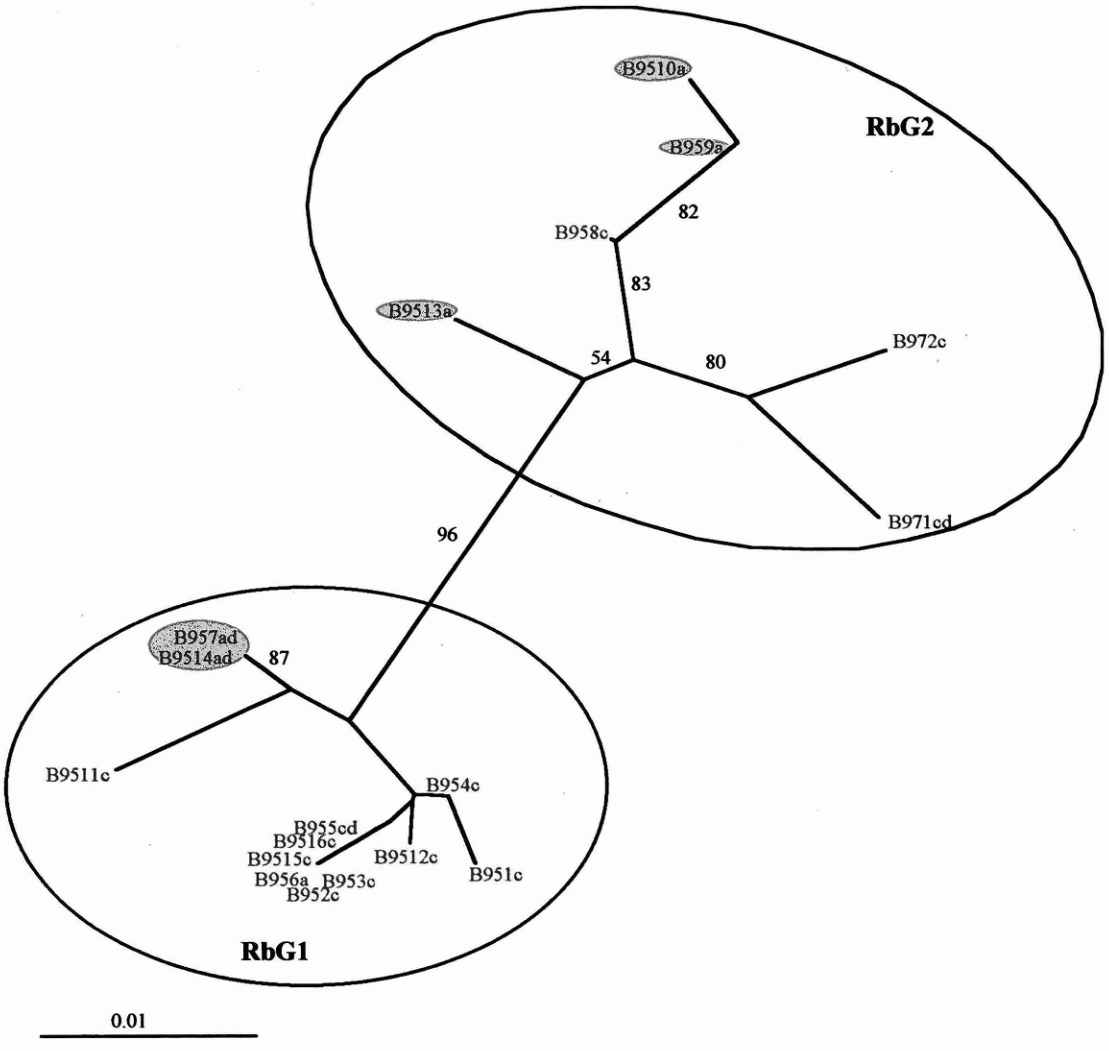
The trees obtained from the analysis of RSV A and B isolates over the three winter seasons studied are shown on the following pages (192-198). The strain types designated here remain constant in all the figs, with all RSV A strains prefixed with RaG, and all RSV B strains prefixed with RbG. Five RSV A strain groups were identified (RaG1-5, fig 5.11) and two RSV b strain groups (RbG1-2, fig 5.12)

Figure 5.11 Community acquired RSV A isolates 95-98



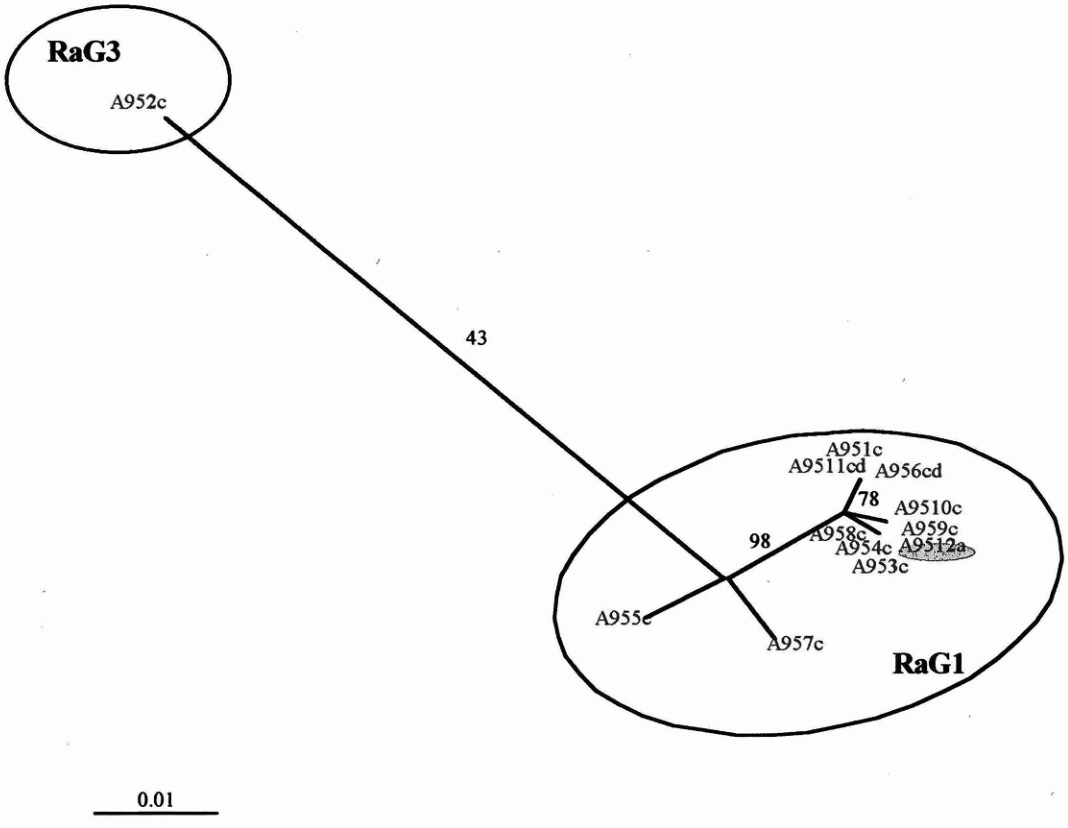
Major clusters are given a strain designation (in bold). The 95/96 season samples are highlighted in red, 96/97 season isolates are highlighted in green, and the 97/98 winter season isolates are highlighted in blue. The isolates obtained from adults are shown circled in grey. Trees were bootstrapped x100 with values over 70 and those of important other branches also shown.

Figure 5.12 Community acquired RSV B isolates 95-98



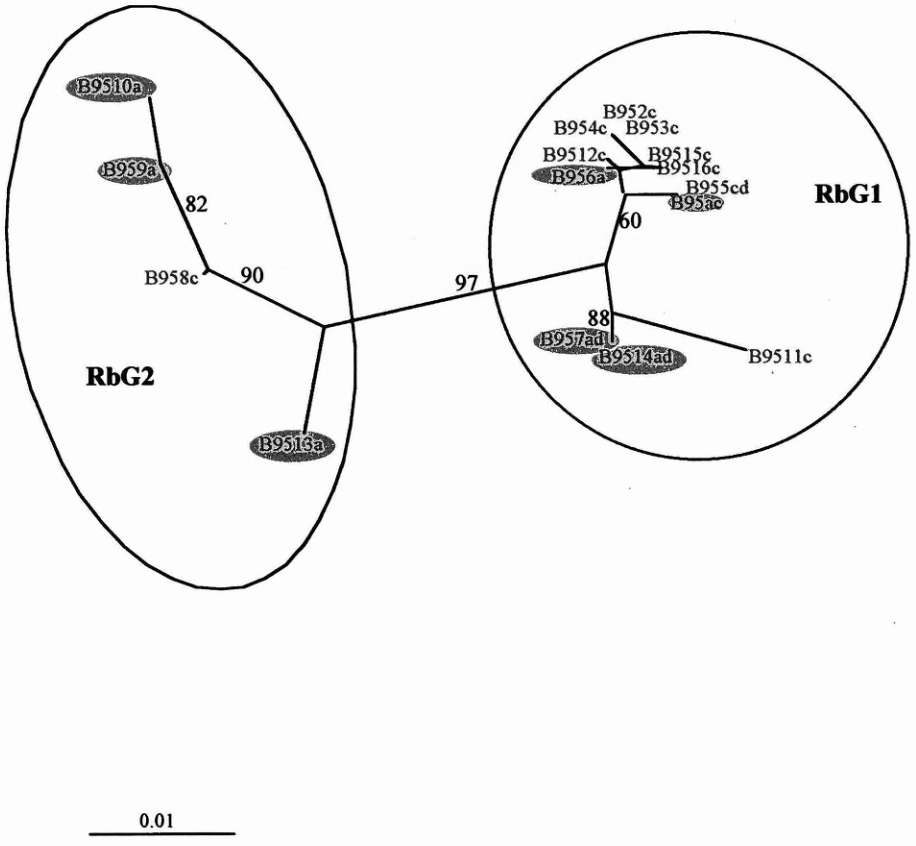
Major clusters are given a strain designation (in bold). The 95/96 season samples are highlighted in red, and the 97/98 winter season isolates are highlighted in blue. The isolates obtained from adults are shown circled in grey. Trees were bootstrapped x100 with values over 70 and those of important other branches also shown.

Figure 5.13 Phylogenetic analysis of community acquired RSV A isolates 95/96 winter season



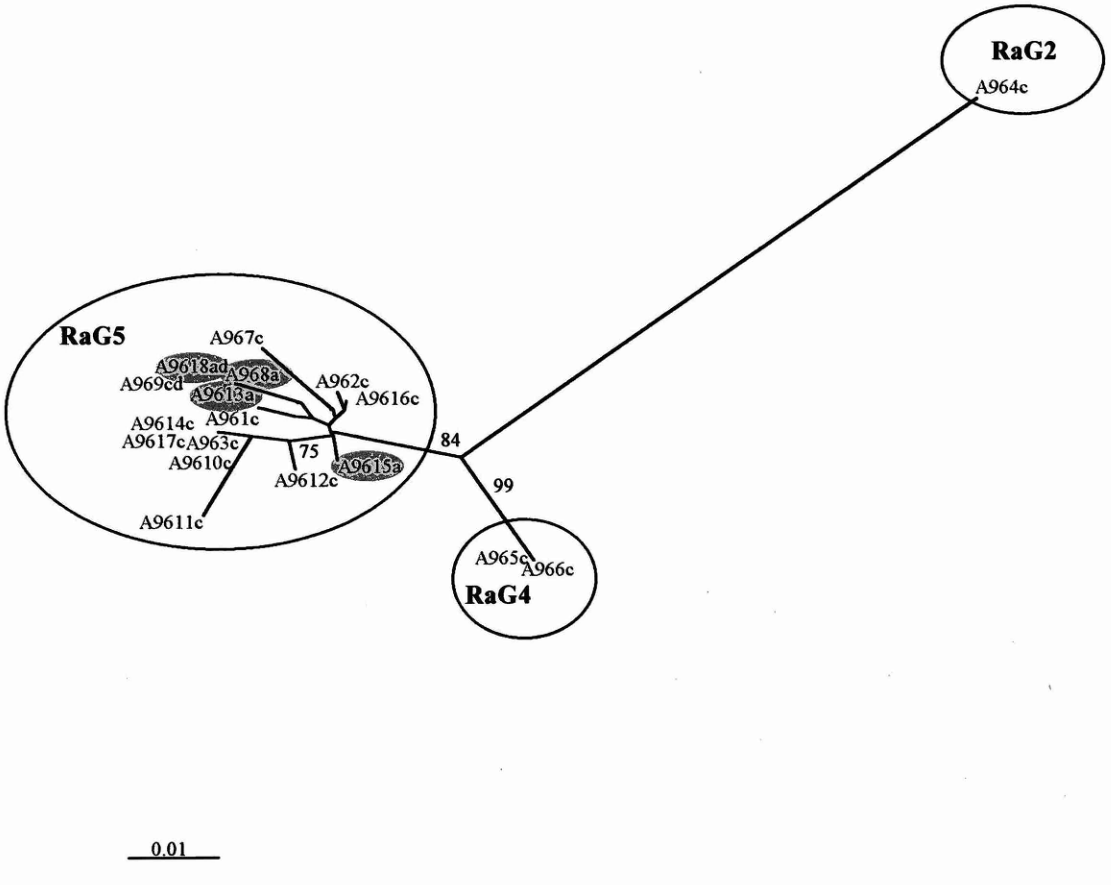
Major clusters are given a strain designation (in bold). The isolates obtained from adults are shown circled in grey. Trees were bootstrapped x100 with values over 70 and those of important other branches also shown.

Figure 5.14 Phylogenetic analysis of community acquired RSV B isolates, 95/96 winter season



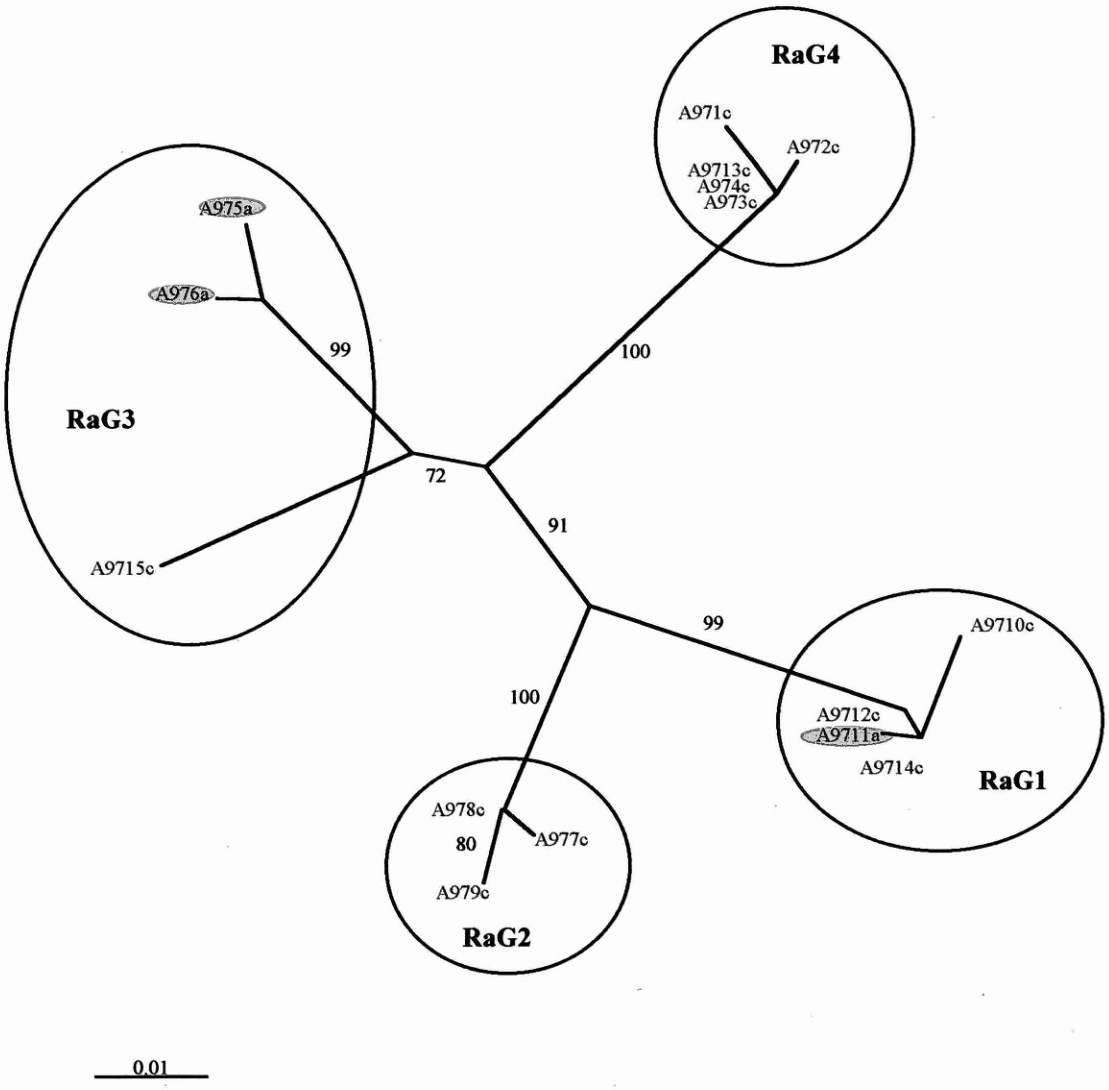
Major clusters are given a strain designation (in bold). The isolates obtained from adults are shown circled in grey. Trees were bootstrapped x100 with values over 70 and those of important other branches also shown.

Figure 5.15 Phylogenetic analysis of community acquired RSV A isolates, 96/97 winter season



Major clusters are given a strain designation (in bold). The isolates obtained from adults are shown circled in grey. Trees were bootstrapped x100 with values over 70 and those of important other branches also shown.

Figure 5.16 Phylogenetic analysis of community acquired RSV A isolates, 97/98 winter season



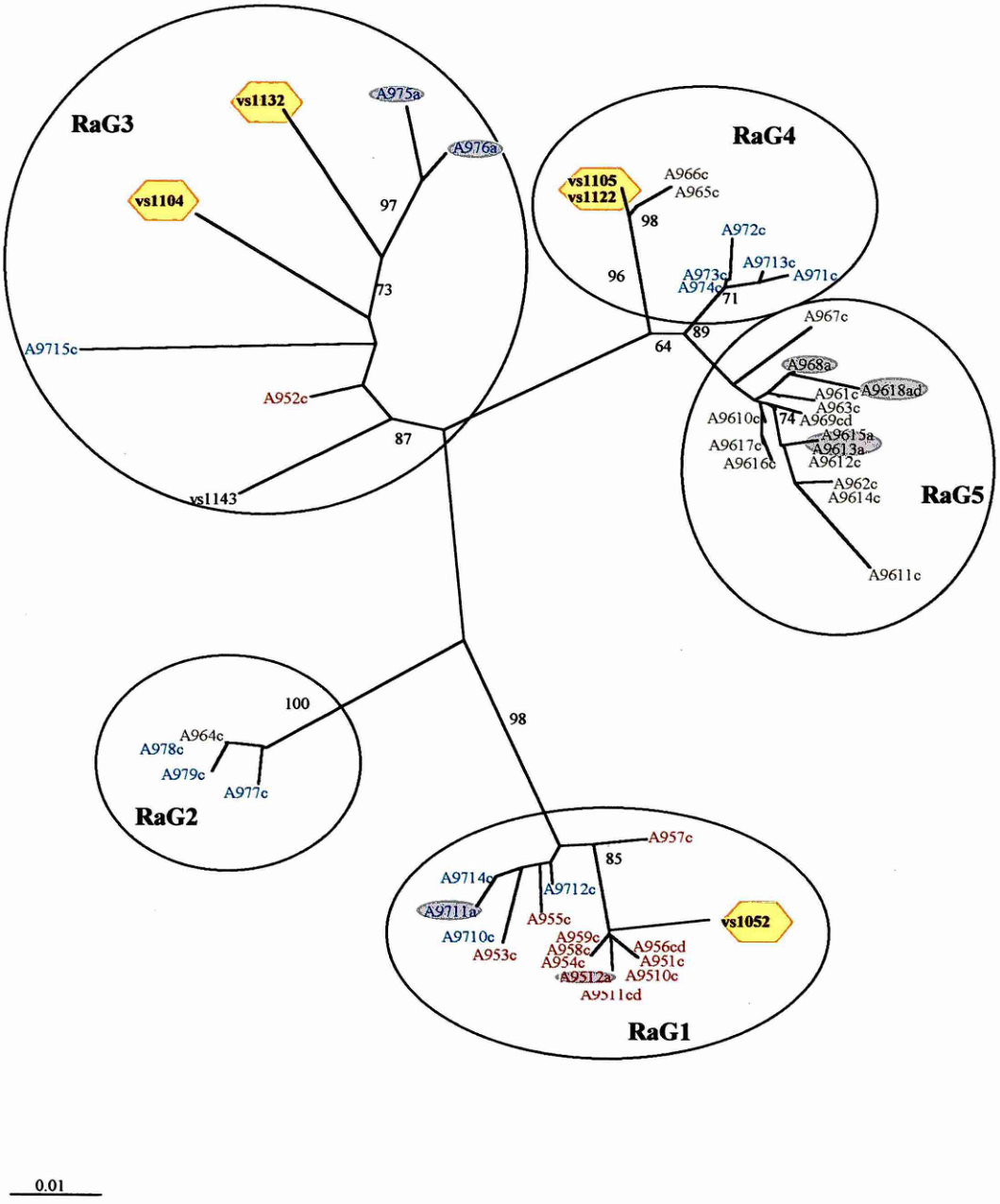
Major clusters are given a strain designation (in bold). The isolate obtained from an adult is shown circled in grey. Trees were bootstrapped x100 with values over 70 and those of important other branches also shown.

RSV isolates were obtained from Dr. P. Cane from hospitalised infants within the Birmingham area whose strain type had already being assigned by restriction enzyme digestion of amplicons of the G and N gene (22). These isolates were amplified and sequenced using the RSV G primers described above (Table 5.4). Comparison of the data from these samples with that from other samples obtained from these seasons was conducted for both RSV A (fig 5.17) and RSV B (fig 5.18).

Table 5.4 Table of isolates donated from Birmingham

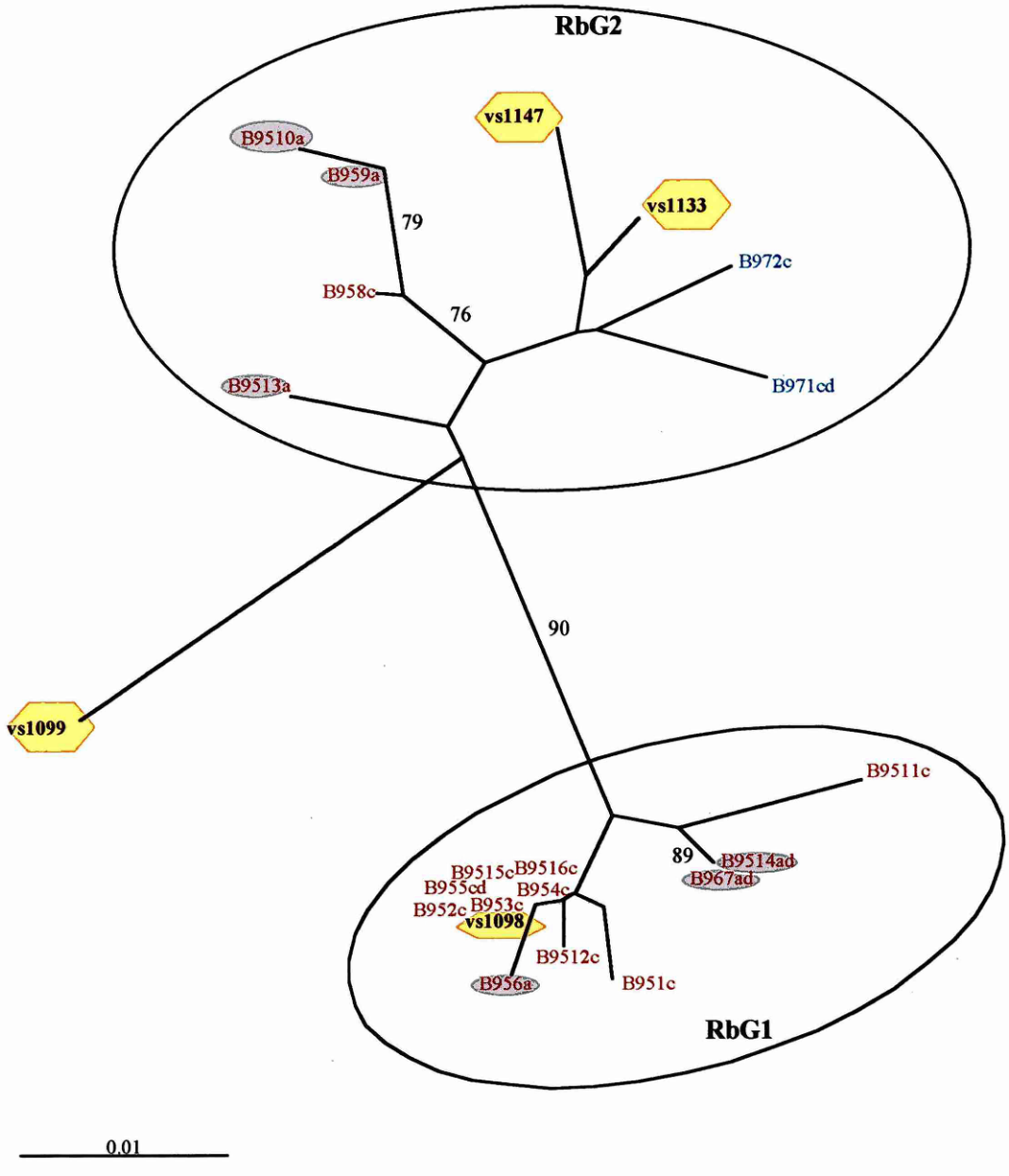
Birmingham Stock Number	Isolate Designation	Epidemic	Subtype	Strain Type
vs1052	Birm/9387/95	95/96	A	SHL-2
vs1098	Birm/9086/95	95/96	B	NP3
vs1099	Birm/8196/95	95/96	B	NP3
vs1104	Birm/12507/96	96/97	A	Novel
vs1105	Birm/12344/96	96/97	A	SHL1/3/4
vs1122	Birm/12291/96	96/97	A	SHL1/3/4
vs1132	Birm/16590/95	97/98	A	SHL1/3/4
vs1133	Birm/16723/97	97/98	B	NP3
vs1143	Birm/18001/97	97/98	A	SHL-6 (Probably)
vs1147	Birm/16099/97	97/98	B	NP3

Figure 5.17 Community acquired RSV A isolates 95-98 analysed with Birmingham RSV A isolates



Major clusters are given a strain designation (in bold). The 95/96 season samples are highlighted in red, 96/97 season isolates are highlighted in green, and the 97/98 winter season isolates are highlighted in blue. The isolates obtained from adults are shown circled in grey. Isolates from Birmingham are prefixed with vs and highlighted with yellow. Trees were bootstrapped x100 with values over 70 and those of important other branches also shown.

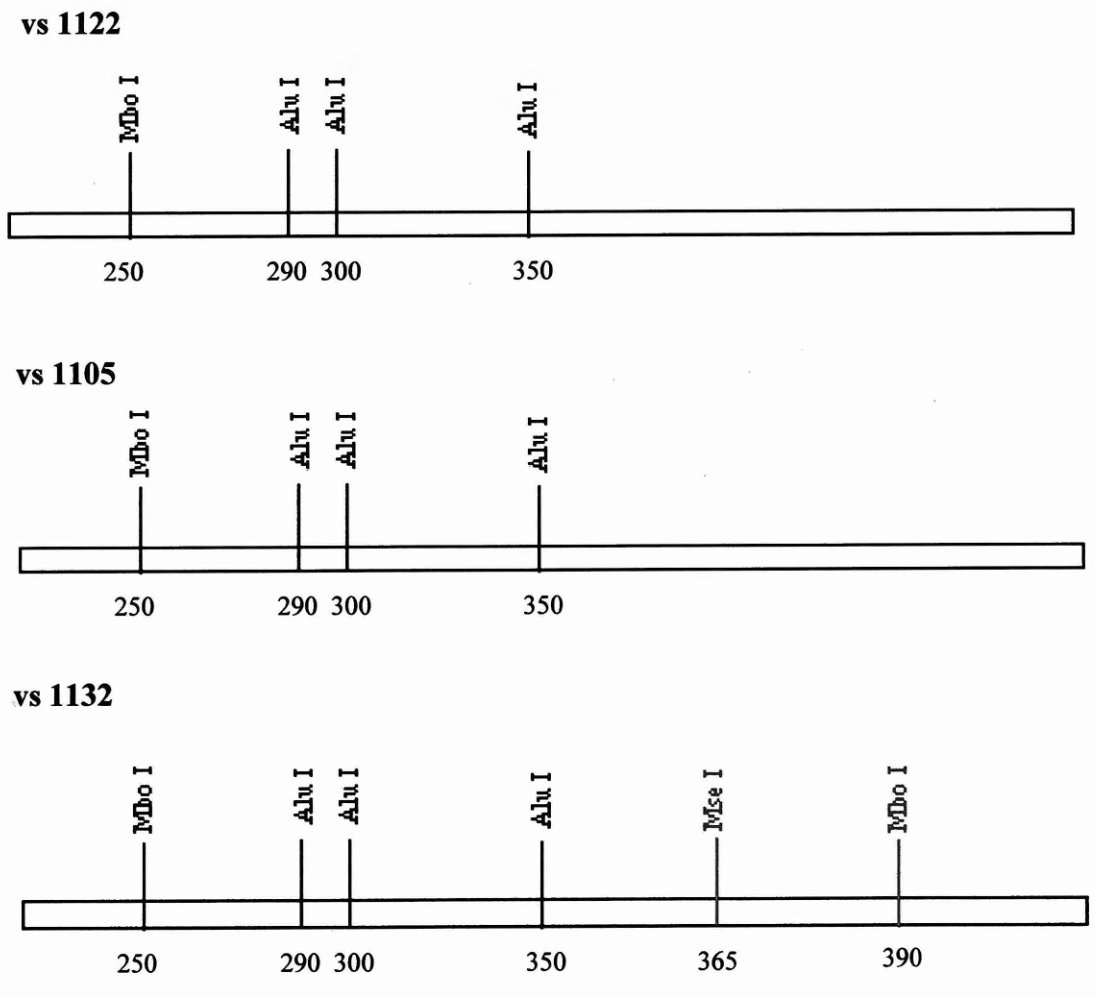
Figure 5.18 Community acquired RSV B isolates 95-98 analysed with RSV B isolates from Birmingham



Major clusters are given a strain designation (in bold). The 95/96 season samples are highlighted in red, and the 97/98 winter season isolates are highlighted in blue. The isolates obtained from adults are shown circled in grey. Isolates from Birmingham are prefixed with vs and highlighted in yellow. Trees were bootstrapped x100 with values over 70 and those of important other branches also shown

Three of the RSV A isolates from Birmingham had been found to be of the same strain designation by restriction analysis (vs1105, vs1122 and vs1132 Table 5.4). Phylogenetic analysis of these strains assigned one of these (vs1132) to a different strain group to the other two (fig 5.17). Part of the gene used for the restriction analysis was sequenced by the methods described earlier. The sequences obtained were analysed for restriction enzyme sites using the program Mapdraw (DNAStar). Comparison of these deduced restriction maps (fig 5.19) reveals two extra restriction sites present in the isolate vs1132, which are absent in the isolates vs1105 and vs1122. These sites should have produced different restriction profile distinguishing this isolate from the other two.

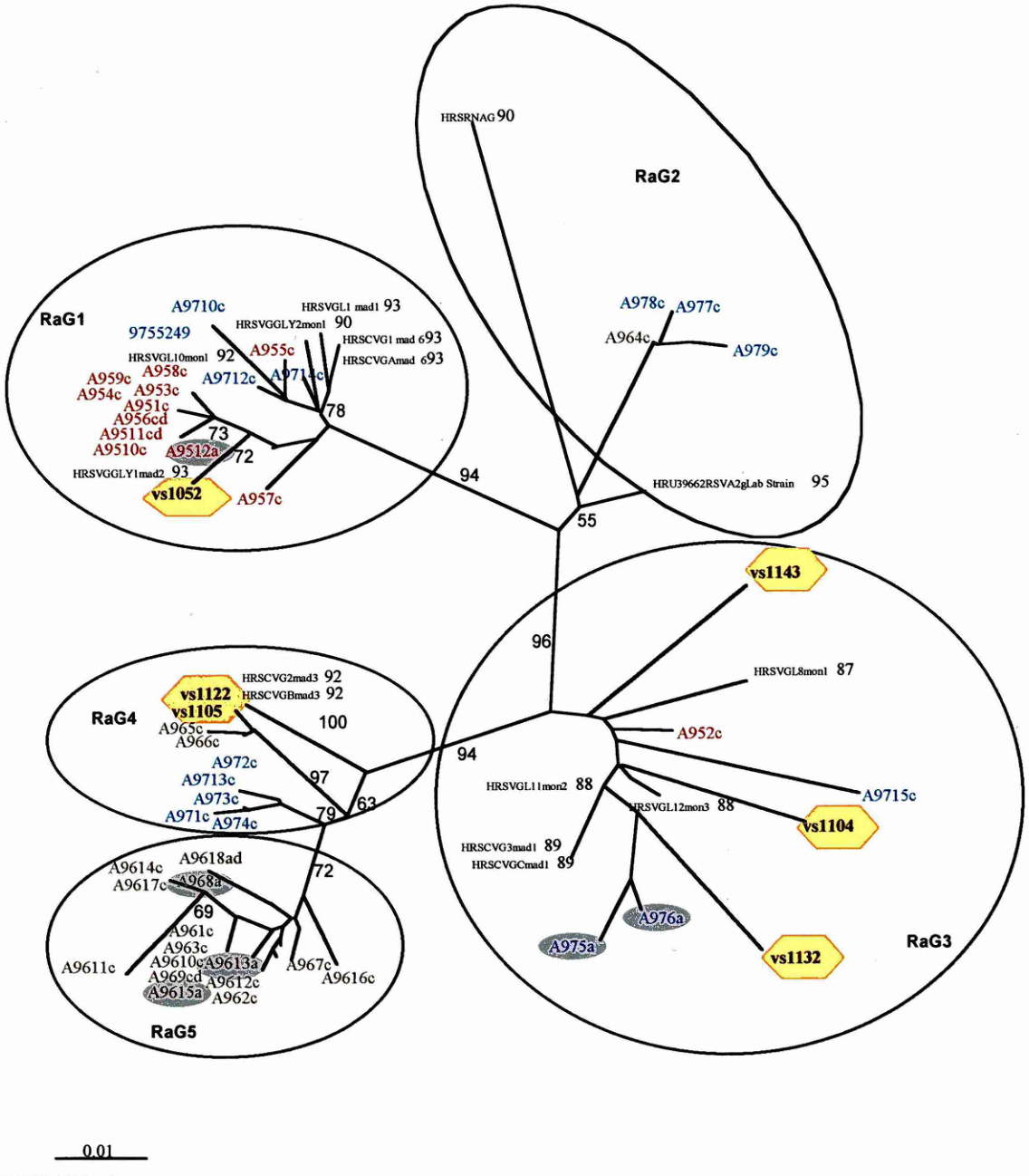
Figure 5.19 Restriction maps of the Birmingham isolates designated strain type SHL-1/3/4



Database sequences were included in the analysis of strain variation for both subgroups A and B (figs 5.20 and 5.21). For RSV A the strain groups were still distinguishable, although some outliers were present mainly from strains which were laboratory adapted (fig 5.20). The RSV B sequences from the database were significantly different from those found in my work (fig 5.21).

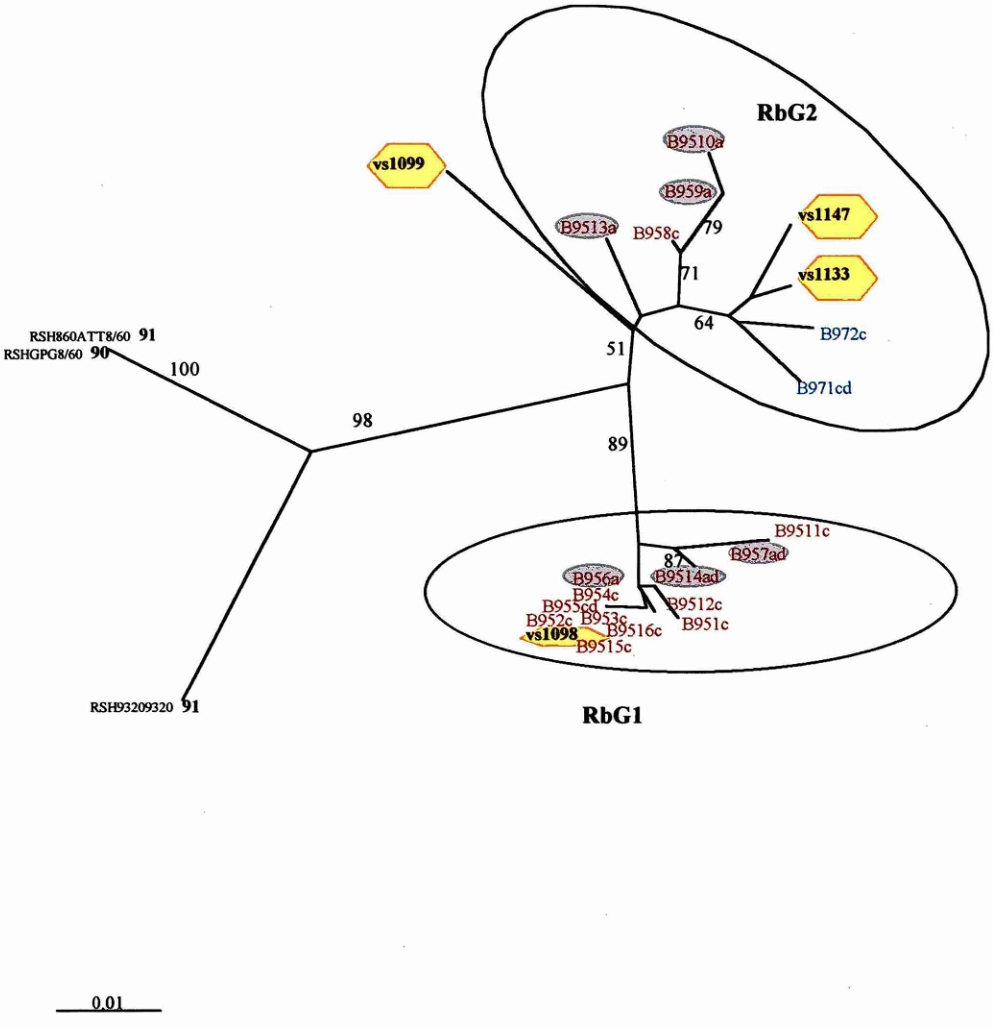
Analysis of the strain types assigned in my work (figs 5.11 to 5.16) revealed a predominance of certain strain types in two winter seasons (95/96 and 96/97), with the 97/98 season having a more representative mixture of circulating strains (fig 5.22 and 5.23).

Figure 5.20 Analysis of community acquired RSV A isolates with available database



Major clusters are given a strain designation (in bold). The 95/96 season samples are highlighted in red, 96/97 season isolates are highlighted in green, and the 97/98 winter season isolates are highlighted in blue. The isolates obtained from adults are shown circled in grey. Isolates from Birmingham are prefixed with vs and highlighted in yellow. Trees were bootstrapped x100 with values over 70 and those of important other branches also shown.

Figure 5.21 Analysis of community acquired RSV B with available database sequences



Major clusters are given a strain designation (in bold). The 95/96 season samples are highlighted in red, and the 97/98 winter season isolates are highlighted in blue. The isolates obtained from adults are shown circled in grey. Isolates from Birmingham are prefixed with vs and highlighted in yellow. Trees were bootstrapped x100 with values over 70 and those of important other branches also shown.

Figure 5.22 Strain type distribution for RSV A winter seasons 95/96, 96/97 and 97/98

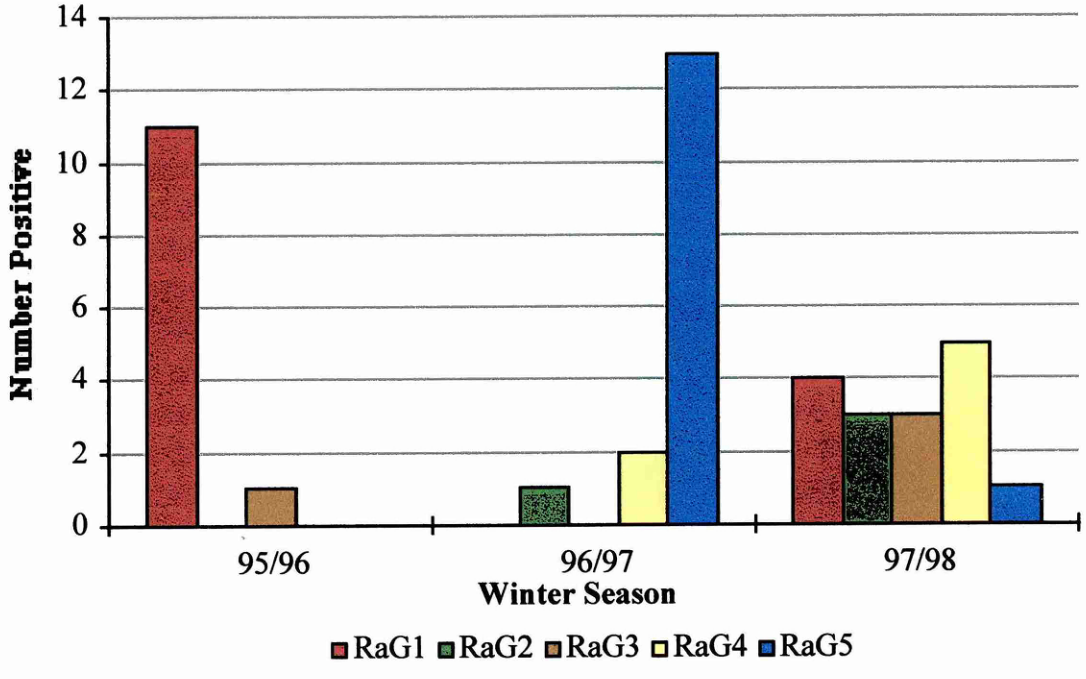
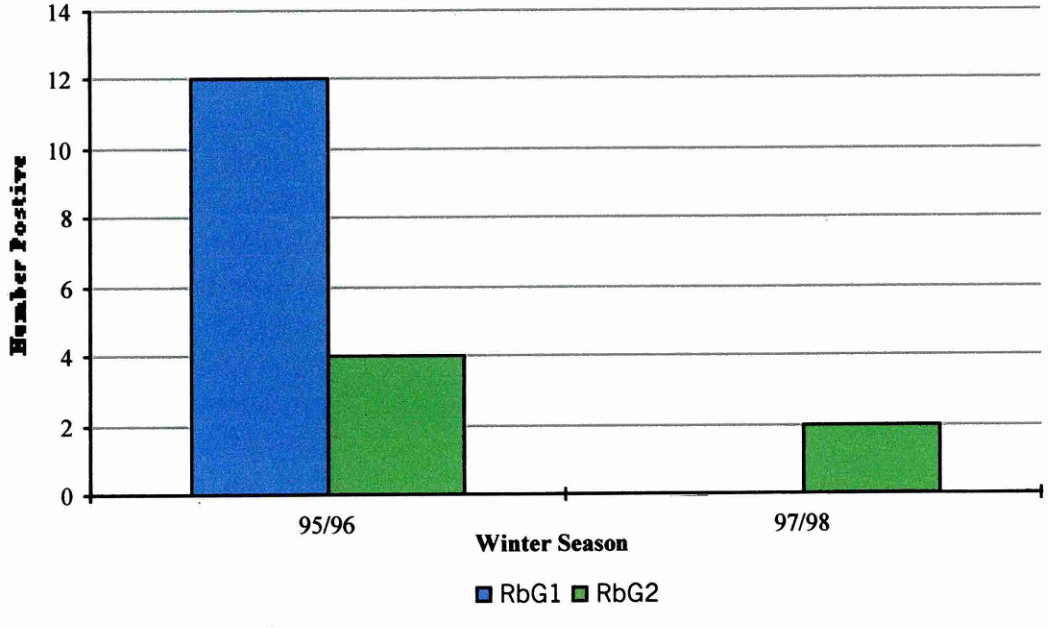


Figure 5.23 Strain type distribution for RSV B winter seasons 95/96 and 97/98



Ts/Tv Ratio

Transition transversion ratios, as calculated in Puzzle, were noted to vary between the different datasets (Table 5.5). In order to investigate if the values obtained were significant the log likelihood values for a range of Ts/Tv ratios were calculated in the program Dnaml (Table 5.6). These values were then subjected to likelihood ratio tests to ascertain the significance of the original Ts/Tv. The results indicated that the 95/96 RSV A dataset (Ts/Tv 3.99) may have been significantly different from the 96/97 RSV A dataset (Ts/Tv 1.91), but more extensive testing would be required to confirm this (Tables 5.5 and 5.6). This would provide more evidence for a selective pressure on this region of the genome.

Table 5.5 Ts/Tv ratios as calculated in the program Puzzle

Group (No of Seqs)	Ts/Tv	Pyrimidine Ts/ Purine Ts	No. constant sites	A (%)	C (%)	G (%)	T (%)
RSV A 95 (12)	2.77	1.17	271 (90.3%)	42.4	33.5	9.8	14.4
RSV B 95 (16)	1.42	1.15	244 (92.8%)	45.3	34.1	8.2	12.5
All 96/97 (18)	1.91	1.02	221 (85.7%)	41.3	37.3	10.1	11.4
97/98RSV A (15)	3.99	1.14	210 (81.4%)	41.4	35.7	9.9	13.0
RSV 95-98 17.5.99 (63)	1.32	1.10	109 (42.2%)	42.8	35	9.5	12.8
Birmingham Isolates (10)	2.38	1.09	126 (48.8%)	43.6	34.4	9.2	12.8

Values calculated in the program Puzzle

Table 5.6 Log likelihood values as calculated in the program Dnaml over a range of Ts/Tv values

	Log Likelihood Values for diferent Ts/Tv ratios*				
Dataset	**0.5**	**1.5**	**2.5**	**3.5**	**4.5**
95/96 RSV A	-463.13	-453.62	-451.79	-451.37	-451.42
95/96 RSV B	-459.75	-455.66	-456.10	-457.10	-458.15
96/97 RSV A	-590.58	-580.05	-580.11	-581.46	-583.38
97/98 RSV A	-648.11	-630.10	-627.05	-626.65	-627.06
All vrd RSV A	-948.16	-923.70	-921.17	-922.48	-924.76
All vrd RSV B	-502.07	-495.68	-495.61	-496.42	-497.44

*Calculated in the program Dnaml

Visual inspection of the amino acid alignments from the RSV A and RSV B isolates from all three winter seasons was done (Alignments in Appendix 2 to 5). No patterns were observed with regard to amino acid changes. The RSV B strains had two potential glycoslylation sites whereas the RSV A strains had 3 potential glycoslylation sites (with the exception of A975c which had 4).

The non-synonyous/synonyous ratio (Ka/Ks) was calculated for both the RSV A and RSV B datasets using the program MEGA (Table 5.7).

Table 5.7 Non-synonyous to synonyous changes in the RSV A and RSV B datasets

Dataset	Ka *	Ks *	Ka/Ks
RSV A	0.0598	0.0900	0.66
RSVB	0.0162	0.0584	0.27

*Calculated in the program MEGA

It would appear that there is more selective pressure for RSV A than RSV B, as there were more non-synonymous changes in the RSV A dataset than in the RSV B dataset.

Discussion

RSV G PCR

Primers were designed to amplify part of the first variable region of the G gene of RSV subtypes A and B. This region allows the differentiation of isolates into strains and allows comparison of circulating strains for the three winter seasons studied in this work. There are other published reports of primers that amplify the RSV G gene (22, 191). However, most of those protocols require high copy number, good quality samples, for instance isolates grown from clinical samples. It was not possible in my work to culture most of the RSV positive samples. Growth of the previously identified isolates was attempted, but recovery was poor presumably due to the lag in time between receipt and innoculation, which was at least one year. Ideally, upon receipt of a sample, an aliquot should immediately be placed in tissue culture which should allow better recovery of the virus. Therefore, although the previously published primers were tested in my work, they were not sensitive enough to recover RSV sequences from the nose and throat swabs available for testing in this laboratory. To overcome this, primers were designed to amplify the first variable region of the RSV G gene and which were capable of detecting virus from clinical samples. In order to assess other techniques for analysis of the G gene I visited the RSV group in CDC Atlanta to learn the techniques used in Dr. Larry Anderson's laboratory. In the short time short there I managed to assess amplification techniques used for RSV G gene sequencing both with high quality samples and with low quality samples from Colindale. A comparison of the CDC techniques with my strategy (fig 5.3 and 5.5) highlighted the different approaches necessary for different sample types: no amplification from the low quality samples with the methods used by CDC was seen. In contrast, tests of various combinations of primers showed that a nested reaction using primers G2F, F95R, G152F and G508R (Table 5.2) gave acceptable results from the sample types available for use in my work.

RSV G sample analysis

Samples which were identified as RSV positive from the winter seasons 95/96, 96/97 and 97/98 were planned to be sequenced but it was clear from initial amplification reactions that there was a lower positivity rate than expected. The reasons for this were investigated. Some degradation of the samples was expected as the samples had been posted to the laboratory and had been tested before storage. All of the samples had been through at least one cycle of thawing and re-freezing before being defrosted for PCR sequencing, with the majority having more than two cycles of defrosting and refreezing. The loss of RNA was seen for both viral (fig 5.7) and cellular targets (fig 5.6).

It was also apparent that the RSV G primers were not amplifying as many samples as the multiplex primers (fig 5.7), although the RSV G primers were shown to be as sensitive as the multiplex RSV primers on laboratory adapted material (fig 5.5). The testing of sensitivity was conducted on laboratory adapted viral strains. It is possible that the currently circulating strains differ in sequence from the laboratory strains, and from those deposited in the Genbank Sequence Database from which the sequences were obtained for primer design. It would appear that a combination of degraded samples and inefficient priming was responsible for the low amplification efficiency. Another possibility is that certain strain types were not amplified due to differences in one of the primer binding sites. Sequence analysis of the RSV from which data could be obtained reveals that different strains were circulating in all of the years studied (Table 5.3), excluding the RSV B isolates from 96/97 season. The low number (10 in all) of RSV B isolates from the 96/97 season accounts for the inability to recover any virus for sequence analysis. Representative strains from Birmingham were also amplified and sequenced with this method. Interestingly these data do not support the suggestion that some virus strains could not amplified because of primer mismatch, but this possibility cannot be entirely ruled out.

Another explanation for the differences in detection of RSV between the multiplex primers and the RSV G primers is differential expression of RSV genes. For the non-segmented negative stranded RNA virus VSV, it has been shown that the amount of mRNA which is transcribed depends upon the proximity of the genes to the 3' end of the genome (254). The

multiplex primers are located in the N gene close to the genome 3' end, whereas the G gene is distant from the 3' end. Wertz *et al* (1998) has shown a 15 fold decrease in gene product by moving the gene one postion further from the 3' end (254). Whether this assay was measuring mRNA or viral RNA was not tested as it was presumed that vRNA was being detected, but if mRNA was being measured a similar phenomenon may provide an explanation for the observed difference in detection between the two assays used in my work.

Resolution of this problem would require redesign of the primers, and sequencing of the entire RSV G gene for the currently circulating strains. Such new sequence information may reveal which primer or primers were not working well. It is also possible that further optimisation of the amplification would improve the detection rate. For this, testing of the primers would need to be both with laboratory adapted and currently circulating strains of RSV.

Further work for RSV PCR

Other improvements to the techniques used in my work could involve proof reading enzymes to reduce *Taq* polymerase induced errors, although reports upon the effectiveness of this strategy are contradictory (16, 35). Cloning PCR amplicons could also be done to reduce the effect of quasispecies variation, although the sequences obtained for most of the samples were clean (good quality, high signal intensity). Some sequences that were poor quality and were not repeated (Table 5.3), and may be due to mixed population of virus in the sample. Cloning of amplicons and subsequent sequence analysis of them would reveal if this were the case. Comparison of sequences obtained directly from the sample, and sequences obtained from the same isolate passaged for three weeks in tissue culture showed that the same majority sequence was present. It is likely that the predominant sequence species from each virus was determined and analysed in my work. Cloning of PCR products would also show whether any infections with RSV were with mixed strains. As some infections were of mixed subtype, this could be a possibility. Time was a constraint during this work, and sequencing of the amplicons obtained was pursued rather than optimisation of the amplification reaction.

There were only a few RSV G sequences available for my PCR primer design. Additionally many of these were deposited over five years ago in Genbank. More recently, other sequences in of the RSV G genes have become available, but analysis of these has not revealed any potential problems which could give rise to primer mismatch. The limitations on primer location for this region are great, due to its variability, and although primers were tested which were located in the constant region, these did not prove to be as sensitive as those finally chosen for the work. Future refinements to the amplification protocol may exploit primers which are subtype specific. However this approach would either require knowledge of the subtype before testing, or would require a multiplex assay. The outer primers were designed such that they amplify all of G gene. An approach which used these as an inner pair of primers in a nested reaction would allow analysis of the entire G gene. This would, in turn, provide more comprehensive data about the sequence of the isolate.

This laboratory was not able to culture any of the RSV samples upon receipt, so all analysis was conducted in retrospect. Culture was performed retrospectively for some of the samples, but this approach was not pursued for all of the viruses as it was clear that it would not necessarily be helpful (fig 5.7).

Recovery of RNA from stored samples

The recovery of virus from the samples declined progressively for each winter season studied (with respect to the amount of RSV detected originally by multiplex PCR). This was unexpected as the quality of the samples improved (due to less time spent in storage) over the three winter seasons. A drift in virus sequence in one or more of the primer regions may account for the decline in detection, although further investigation of this would be required.

ß-actin RNA was detected in eight of the ten samples tested, this may reflect the quality of the samples. An explanation for the loss of detection in the two samples tested may be degradation of the RNA or that there was insufficient cellular RNA taken during the original swab. The levels of cellular material can vary considerably between swabs (193). As combined nose and throat swabs used for analysis in this work were taken by many

different GPs on different times during the winter season, it is highly likely that the amount of cellular material present in each swab will vary.

Strain determination of RCGP isolates

The results from the amplification of the G gene were not as good as expected (Table 5.3). Nevertheless, sequence information was obtained for 63 specimens and analysis of these has illustrated the circulation pattern for the isolates identified over the three winter seasons. The RSV A isolates can be differentiated into five strains; and the RSV B isolates into two strains (figs 5.14 and 5.15). The predominance of one strain type can be concluded only for RSV A as the numbers for RSV B are two small from seasons other than 95/96. For the first two winter seasons (95/96 and 96/97) one RSV A strain type is predominant, although other strain types are present and co-circulating. This is similar to the findings of other groups (22, 25, 88, 157). However, for the last winter season (97/98) more variation was seen in the circulating strains and the pattern of predominance is not as pronounced as the other two seasons (fig 5.21). This mixture of strain predominance, followed by similar levels of co-circulation of strains was also observed by Fletcher *et al* (1997) who studied the strain variation in two consecutive RSV epidemics in hospitalised infants (82). Another explanation for the lack of predominance of circulating strains for the 97/98 season may be low influenza activity. There may be interaction between the RSV and influenza viruses which results in only the fittest of the RSV viruses being able to establish infection.

Analysis of strains

As bootstrap analysis was unfeasable with maximum likelihood, a comparison of the trees produced by maximum likelihood and neighbor joining was performed (fig 5.10). As no differences were observed between the two trees, neighbor joining was used for all subsequent bootstrap analysis.

The synonyous/nonsynonyous ratio would indicate that there was more selection pressure on the RSV A sequences than on the RSV B sequences. As the sequences analysed here were relatively short (258bp), further analyses into other areas of the genome, particularly conserved regions of the genome would be required before any definite conclusions could

be drawn. As the amount of RSV B circulating in the community was lower than that of the RSV A (as determined by multiplex PCR – Chapter 3), it is likely that RSV A was under a greater selection pressure.

It can be seen that the strains recovered from adults are co-circulating with those recovered from children in the same winter season, and do not form separate lineages. As the isolates studied were all from community acquired infections this is what was expected, as presumably transmission will occur between all age groups. The samples obtained from Birmingham were from infants during the same epidemic periods, and also showed co-circulation of the same strain types as the strains from the general community.

In contrast to the findings of Fletcher *et al* (1997) I could not associate any strain type with a particular symptom type (82). However, indicators of disease severity were not recorded beyond the initial visit to the GP and even this was not always available for analysis. Further investigation is necessary to determine whether particular strain types can be associated with more severe disease in community acquired infections. This would be difficult to measure in these cases due to the necessity of following the progression of the illness in patients in the community. This type of study is inherently more simple when the patient is hospitalised.

The majority of the strains studied were from the Midlands. This probably reflects the sampling bias in this area, especially in the first two winter seasons (Chapter 4). However, isolates from the north and south of the country were obtained and shown to circulate with the same strain types isolated from the midlands in each season.

This work provides the first data from community acquired RSV illness throughout England and is in contrast to the findings from America of Anderson *et al* (1991) (2). They found that the same predominant strains did not co-circulate (2). The two countries differ with respect to the RSV seasonality in that, due to the size of America and the different climates, the RSV season starts at different times in different states. In England the RSV season is more synchronous, probably due to the smaller size of the country.

Birmingham strains

The analysis of the strains from Birmingham shows that they cluster differently by sequencing than by restriction enzyme digestion of RSV G and N amplicons. Analysis of the three subtypes which were reported to be the same strain by restriction enzyme analysis (vs1105, vs1122 and vs1132) revealed two restriction enzyme sites which were different in one of the isolates vs1132 (fig 5.19). It can be seen that this isolate contains two extra restriction enzyme sites compared to the others. It is of course possible that these two restriction enzyme sites may be due to errors in amplification or sequencing (*Taq* induced), or that the restriction enzyme site pattern from an analysis including the SH and G genes would show the pattern associated with the SHL-1/3/4 strain type. Further work is necessary to fully explain the differences seen with the two techniques. This could involve cloning of the PCR product and sequencing several clones to eliminate as far as possible the effect of any *Taq* errors. The characterisation of these isolates with monoclonal antibodies may also provide a method for distinguishing between them. It is interesting to note that the two isolates identified as the same strain type here (vs1105 and vs1122) were from the same season, and the isolate (vs1132) that was different was from a different season. This may reflect differences in genomic sampling between the two techniques used to assign strain type. The strain designation for the RSV B subgroup was performed using restriction enzyme analysis analysis of the N gene so a similar comparison was not possible. Part of the same region was analysed in both assays for the RSV A strains and analysis of restriction enzymes predicted in the sequence was done.

The sequence differences between circulating strains of RSV B and those obtained from the database may be due to the geographical and temporal sources of the strains. The database strains were analysed around 1990/91, and it is not recorded whether they were laboratory adapted strains of the virus or not. The research groups who deposited these sequences are American.

Ts/Tv Ratios

The transistion/transversion ratio may provide some information about the selection pressure on this part of the virus genome. In my work a variation was observed in the Ts/Tv ratio. Of particular interest was the apparent change in RSV A Ts/Tv for the 96/97 winter season as compared with the 95/96 and 97/98 winter seasons (Table 5.5). It has been reported that the lower the value of the Ts/Tv the more evidence for positive selection pressure on that region of the virus genome (186). The RSV G gene may be under positive selection pressure as discussed in this chapter, although my data shows an apparent fluctuation in the Ts/Tv for the different datasets. This would seem to suggest that the selection pressure on this region of the RSV A genome changes yearly. Although I attempted to ascertain whether the changes in the Ts/Tv ratios observed were of significance, the sequence information for the conserved regions of the genome were unavailable for the isolates tested in this work. This would have allowed a comparison between the different regions in the same datasets, and allowed a more meaningful analysis. However the results obtained would indicate that the differences between the higher and lower Ts/Tv ratios may be significant. It would be interesting if the selection pressures were fluctuating for this region in different winter seasons.

The reasons for the alteration in selection pressure for this region are undoubtedly complex. It could be speculated that these differences are related to the strain of the virus, as the predominant strain for the 95/96 season (RaG1) was different from the predominant strain for the 96/97 season (RaG5). The 97/98 winter season had representative strains in most of the groups, but still had a Ts/Tv ratio similar to the 95/96 RSV winter season. Unfortunately, the numbers of strains seen in each group are too small to allow analysis of the Ts/Tv ratio for the 97/98 winter season. Other factors may account for a change in selection pressure, including the immune status of the population being infected which could affect the selection pressure on the virus. It may be that the predominant strain isolated from the 96/97 winter season was insufficiently different from previous infecting strains and was therefore was partially recognised. Another explanation could be that the amount of circulating virus for each year is reflected in the Ts/Tv values observed.

Further research is required before any conclusions can be made about the significance of the variation of Ts/Tv ratios seen in this work. It is also important to better characterise the strain circulation pattern for the following winter season to be able to determine if the pattern of predominance is the same, and also whether the Ts/Tv ratio changes.

The low G/C content of the G gene region studied here would also support the view that this region of the genome is constantly changing. Visual examination of the amino acid changes within each isolate did not reveal any obvious variation or conservation in amino acid type, however more sophisticated modelling methods would be required to reveal any informative sites. Th alteration of glycoslylation sites in the virus has been reported to be a mechanism for escaping the host immune response (24, 165). The identification of potential glycoslyation sites will provide more information on this possible mechanism.

Appendix 2

RSV A Partial G gene nucleotide alignment

```
RSH1CE AACCACAAAGTCACACCAACAACTGCAATCATACAAGATGCAACAAGCCAGATCAAGAACACAACCCCAACATACCTCACCCAGAATCCT
A951c  .C.A.....T.....................................................G.....
A952c  .............T.........................A.....................C
A953c  ...A.....T.....................................................G.....
A954c  ...A.....T.....................................................G.....
A955c  ...A.....T.....................................................G.....C
A956cd .C.A.....T.....................................................G.....
A957c  ...A.....T.....................................................G.....
A958c  ...A.....T.....................................................G.....
A959c  ...A.....T.....................................................G.....
A9510c ...A.....T.....................................................G.....
A9511cd.C.A.....T.....................................................G.....
A9512a ...A.....T.....................................................G.....
A961c  .........C.................C.....................................T.....C
A962c  .........C.................C.....................................T.....C
A963c  .........C.................C.....................................T.....C
A964c  .............T.....................................................G....C
A965c  .............T...........C.....................................................C
A966c  .............T...........C.....................................................C
A967c  ............................C.....................................G.T.....C
A968a  .........C.................C.....................................T.....C
A969cd .........C.................C.....................................T.....C
A9610c .........C...........G.....C.....................................T.....C
A9611c .........C...........G.....C.....G...............................T.....C
A9612c ...A.....C...........G.....C.....................................T.....C
A9613a .........C.................C.....................................T.....C
A9614c .........C...........G.....C.....................................T.....C
A9615a .........C.................C.....................................T.....C
A9616c .........C.................C.....................................T.....C
A9617c .........C...........G.....C.....................................T.....C
A9618ad.........C.................C.....................................T.....C
A971c  ......................C.....C.....................................C
A972c  ..........................T.C.....................................C
A973c  ..........................C.....................................C
A974c  ..........................C.....................................C
A975a  .......GG.......T.........................C.....................C
A976a  .............T.........................C.....................C
A977c  ............GT.....................................................G....C
A978c  .............T.....................................................G....C
A979c  .............T.....................................................G....C
A9710c ...A.....T.....................................G.....C
A9712c ...A.....T.....................................G.....C
A9711a ...A.....TT....................................G.....C
A9713c ......................C.....C.....................................C
A9714c ...A.....T.....................................G.....C
A9715c .............T.....A.....................................A.....C
```

Nucleotide alignment of the partial G gene sequences in this work from all of the RSV A strains 95-98 with Genbank reference strain RSH1CE (bases 4837-4927)

Appendix 2 RSV A Partial G gene nucleotide alignment continued

```
RSH1CE CAGCTTGGAATCAGTCCCTCTAATCCGTCTGAAATTACATCACAAATCACCACCATACTAGCTTCAACAACACCAGGAGTCAAGTCAACC
A951c  .............CTT..TC....T.....G..C...........C....G...........T.........A.T...G......T.
A952c  .............C.T...C....T...C....C.........CC..........C.............A.T.CTG........
A953c  .............CTT..TC....T....AG..C...........C....G...........T.........A.T...G......T.
A954c  .............CTT..TC....T....AG..C...........C....G...........T.........A.T...G......T.
A955c  .............CTT..TC....T.....G..A...........C....G...........T.........A.T...G......T.
A956cd .............CTT..TC....T.....G..C...........C....G...........T.........A.T...G......T.
A957c  .............CTT..TC....T.....G..C...........C..............T.........A.T...G......T.
A958c  .............CTT..TC....T....AG..C...........C....G...........T.........A.T...G......T.
A959c  .............CTT..TC....T....AG..C...........C....G...........T.........A.T...G......T.
A9510c .............CTT..TC....T.....G..C...........C....G...........T.........A.T...G......T.
A9511cd.............CTT..TC....T.....G..C...........C....G...........T.........A.T...G......T.
A9512a .............CTT..TC....T.....G..C...........C....G...........T.........A.T...G......T.
A961c  .............CTT...C....T........CC.........CC........C..C....CT.G.......TA.T.CTG........
A962c  .............CTT...C....T..G.....CC.........CC........C..C..T.CT.G.......TA.T.GTG........
A963c  .............CTT...C....T........CC.........CC........C..C....CT.G.......TA.T.GTG........
A964c  .............CTT...C....T........C.....................................A.T...G.A......
A965c  .............CTT...C....TA...A...CC.........CC........C..C....CT.G........A.T.GTG........
A966c  .............CTT...C....TA...A...CC.........CC........C..C....CT.G........A.T.GTG........
A967c  .............CTT...C....T........CC.........CC........C..C..T..T.G.......TA.T.GTG........
A968a  .............CTT...C....T........CC.........CC........C..C..T.CT.G.......TA.T.CTG........
A969cd .............CTT...C....T........CC.........CC........C..C....CT.G.......TA.T.CTG........
A9610c .............CTT...C....T........CC.........CC........C..C....CT.G.......TA.T.GTG........
A9611c .............CTT...C....T........CC.........CC........C..C....CT.G.......TA.T.GTG....T...
A9612c .............CTT...C....T........CC.........CC........C..C....CT.G.......TA.T.GTG........
A9613a .............CTT...C....T........CC.........CC........C..C..T.CT.G.......TA.T.GTG........
A9614c .............CTT...C....T........CC.........CC........C..C....CT.G.......TA.T.GTG...T....
A9615a .............CTT...C....T........CC.........CC........C..C....GT.G.......TA.T.GTG........
A9616c .............CTT...C....T..G.....CC.........CC........C..C....CT.G.......TA.T.GTG........
A9617c .............CTT...C....T........CC.........CC........C..C....CT.G.......TA.T.GTG........
A9618ad.............CTTT..C....T........CC.........CC........C..C..T.GT.G.......TA.T.CTG........
A971c  .............CTT...C....T..T.....CC.........CC........C..C..T.CT.G........A.T.CTG........
A972c  .............CTT...C....T..T.....CC.........CC........C..C....CT.G........A.T.CTG........
A973c  .............CTT...C....T..T.....CC.........CC........C..C....CT.G........A.T.CTG........
A974c  .............CTT...C....T..T.....CC.........CC........C..C....CT.G........A.T.CTG........
A975a  .............CTT...C....T...C....C..........CCT.....................A.T.CTG........
A976a  .............CTT...C....T...C....C..........CCT.....................A.T.CTG....T...
A977c  .............CTT...C....T........C.....................................A.T...G.A......
A978c  .............CTT...C....T........C.....................................A.T...G.A......
A979c  .............CTT...C....T........C.....................................A.T...G.A......
A9710c .............CTT..TC....T.....G..C..T.........C....G...........T.........A.T..TG......T.
A9712c .............CTT..TC....T.....G..C..T.........C....G...........T.........A.T...G......T.
A9711a .............CTT..TC....T.....G..C..T.........C....G...........T.........A.T...G......T.
A9713c .............CTT...C....T..T.....CC.........CC........C..C....CT.G........A.T.CTG........
A9714c .............CTT..TC....T.....G..C..T.........C....G...........T.........A.T...G......T.
A9715c .............CTT........T...C....C..........CC.....................G.....A.T.C.G........
```

Nucleotide alignment of the partial G gene sequences in this work from all of the RSV A strains 95-98 with Genbank reference strain RSH1CE (bases 4928-5018)

Appendix 2 RSV A Partial G gene nucleotide alignment continued

```
RSH1CE CTGCAATCCACAACAGTCAAGACCAAAAACACAACAACAACTCAAACACAACCCAGCAAGCCCACCACAAAACAACGC
A951c  .C.....T.............T.......T.........C....T.....................................
A952c  .CA.....................................C....T.....................................
A953c  .C.....T.............T.......T.........C....T.....................................
A954c  .C.....T.............T.......T.........C....T.....................................
A955c  ........T.................................C....T.....................................
A956cd .C.....T.............T.......T.........C....T.....................................
A957c  ........T.................................C....T........A.............................
A958c  .C.....T.............T.......T.........C....T.....................................
A959c  .C.....T.............T.......T.........C....T.....................................
A9510c .C.....T.............T.......T....C....C....T.....................................
A9511cd.C.....T.............T.......T.........C....T.....................................
A9512a .C.....T.............T.......T....C....C....T.....................................
A961c  .CA.....................................C....T.....................................
A962c  .CA.....................................C....T.....................................
A963c  .CA.....................................C....T.....................................
A964c  ....T..........A.....................T..C....T.....................................
A965c  TCA.....................................C....T............T.........................
A966c  TCA.....................................C....T............T.........................
A967c  .CA.....................................C....T.....................................
A968a  .CA.....................................C....T.....................................
A969cd .CA.....................................C....T............T.........................
A9610c .CA.....................................C....T.....................................
A9611c .CA...................A.................C....TT....................................
A9612c .CA.....................................C....T.....................................
A9613a .CA.....................................C....T.....................................
A9614c .CA...................A.................C....T.....................................
A9615a .CA.....................................C....T.....................................
A9616c .CA.....................................C....T.....................................
A9617c .CA.....................................C....T.....................................
A9618ad.CA.....................................C....T.....................................
A971c  .CA.....................................C....T.....................................
A972c  .CA.....................................C....T.....................................
A973c  .CA.....................................C....T.....................................
A974c  .CA.....................................C....T.....................................
A975a  .CA...................T.................C....T......T.....A.........................
A976a  .CA...................T.................C....T......T.....A.........................
A977c  ....T.................................T..C....T.....................................
A978c  ....T.................................T..C....T.....................................
A979c  ....T..........A.....................T..C....T............T.........................
A9710c ........T..T.............................C....T.....................................C......
A9712c .....................................C....T.....................................
A9711a ........T.....................................C....T.....................................
A9713c .CA.....................................C....T.....................................
A9714c ........T.....................................C....T.....................................
A9715c .CA.T.....................T.........C....T......T.....................................
```

Nucleotide alignment of the partial G gene sequences in this work from all of the RSV A strains 95-98 with Genbank reference strain RSH1CE (bases 5019-5097)

Appendix 3

RSV A Partial G gene amino acid alignment

```
RSH1CE NHKVTPTTAIIQDATSQIKNTTPTYLTQNPQLGISPSNPSEITSQITTILASTTPGVKSTLQSTTVKTKNTTTTQTQPSKPTTKQR
A951c  TN...L.............................FF.L.GT...T.A...L...S.E.IP.........I....I.........
A952c  .....L.........N...................L..L..T...P...P.....SAE..P.............I.........
A953c  .N...L.............................FF.L.RT...T.A...L...S.E.IP.........I....I.........
A954c  .N...L.............................FF.L.RT...T.A...L...S.E.IP.........I....I.........
A955c  .N...L.............................FF.L.GN...T.A...L...S.E.I..............I.........
A956cd TN...L.............................FF.L.GT...T.A...L...S.E.IP.........I....I.........
A957c  .N...L.............................FF.L.GT...T.....L...S.E.I..............I..N.......
A958c  .N...L.............................FF.L.RT...T.A...L...S.E.IP.........I....I.........
A959c  .N...L.............................FF.L.RT...T.A...L...S.E.IP.........I....I.........
A9510c .N...L.............................FF.L.GT...T.A...L...S.E.IP.........I.P..I.........
A9511cdTN...L.............................FF.L.GT...T.A...L...S.E.IP.........I....I.........
A9512a .N...L.............................FF.L.GT...T.A...L...S.E.IP.........I.P..I.........
A961c  ...A......T..............I........F..L..T...P..TP.LA..SAE..P.............I.........
A962c  ...A......T..............I........F..LC.T...P..TPVLA..SGE..P.............I.........
A963c  ...A......T..............I........F..L..T...P..TP.LA..SGE..P.............I.........
A964c  .....L.....................D......F..L..T.............S.E...L...I.........I.........
A965c  .....L....T.......................F..L.KT...P..TP.LA..SGE..S.............I....S.....
A966c  .....L....T.......................F..L.KT...P..TP.LA..SGE..S.............I....S.....
A967c  ..........T..............I........F..L..T...P..TPVLA..SGE..P.............I.........
A968a  ...A......T..............I........F..L..T...P..TPVLA..SAE..P.............I.........
A969cd ...A......T..............I........F..L..T...P..TP.LA..SAE..P.............I....S.....
A9610c ...A....G.T..............I........F..L..T...P..TP.LA..SGE..P.............I.........
A9611c ...A....G.T..G...........I........F..L..T...P..TP.LA..SGE..P......N.......I.........
A9612c .N.A....G.T..............I........F..L..T...P..TP.LA..SGE..P.............I.........
A9613a ...A......T..............I........F..L..T...P..TPVLA..SGE..P.............I.........
A9614c ...A....G.T..............I........F..L..T...P..TP.LA..SGEL.P......N.......I.........
A9615a ...A......T..............I........F..L..T...P..TP.VA..SGE..P.............I.........
A9616c ...A......T..............I........F..LC.T...P..TP.LA..SGE..P.............I.........
A9617c ...A....G.T..............I........F..L..T...P..TP.LA..SGE..P.............I.........
A9618ad...A......T..............I........F..L..T...P..TPVVA..SAE..P.............I.........
A971c  ..........T.......................F..LF.T...P..TPVLA..SAE..P.............I.........
A972c  ..........T.......................F..LF.T...P..TP.LA..SAE..P.............I.........
A973c  ..........T.......................F..LF.T...P..TP.LA..SAE..P.............I.........
A974c  ..........T.......................F..LF.T...P..TP.LA..SAE..P.............I.........
A975a  ..R..L.........T..................F..L..T...P.........SAE..P......I.......I.........
A976a  .....L.........T..................F..L..T...P.........SAE..P......I.......I.........
A977c  .....V.....................D......F..L..T.............S.E...L.............I.........
A978c  .....L.....................D......F..L..T.............S.E...L.............I.........
A979c  .....L.....................D......F..L..T.............S.E...L...I.........I....S.....
A9710c .N...L.............................FF.L.GTI..T.A...L...S.E.I..............I.......N..
A9712c .N...L.............................FF.L.GTI..T.A...L...S.E.I..............I.........
A9711a .N...L.............................FF.L.GTI..T.A...L...S.E.I..............I.........
A9713c ..........T.......................F..LF.T...P..TP.LA..SAE..P.............I.........
A9714c .N...L.............................FF.L.GTI..T.A...L...S.E.I..............I.........
A9715c .....L.............................F..L..T...P.......A.SAE..PL............I.........
```

Amino acid alignment of the partial G gene sequences in this work from all of the RSV A strains 95-98 with Genbank reference strain RSH1CE (amino acids 1612-1699)

Appendix 4

RSV B Partial G gene nucleotide alignment

```
RSH86  AATCACAAAGTTACACTAACAACAGTTACAGTTCAAACAATAAAAAACCACACTGGAAAAAACATCTCCACCTACCTTACTCAAGTCCCA
B951c  .........................C......TC.....................A..........A....T..............T..
B952c  .........................C......TC.....................A..........A....T..............T..
B953c  .........................C......TC.....................A..........A....T..............T..
B954c  .........................C......TC.....................A..........A....T..............NT..
B955cd .........................C......TC.....................A..........A....T..............T..
B956a  .........................C......TC.....................A..........A....T..............T..
B957ad .........................C......TC.....................A..........A....T..............T..
B958c  ........................G..C.........................A.A..........A....T..............T..
B959a  ........................G..C.........................A.A..........A....T..............TT..
B9510a ........................G..C.........................A.A..........A....T..............TT..
B9511c .........................C......TC.....................A..........A....T..............TT..
B9512c .......N.................C......TC.....................A..........A....T..............AT..
B9513a ........................G..C.........................A..........A....T..............T..
B9514ad.........................C......TC.....................A..........A....T..............T..
B9515c .........................C......TC.....................A..........A....T..............T..
B9516c .........................C......TC.....................A..........A....T..............T..
B971cd ..............G........T..C.............................AG.........A....T..............T..
B972c  ........................T..C.............................AG.........A....T..............T..

RSH86  CCAGAAAGGGTCAACTCATCCAAACAACCCACAACCACATCACCAATCCACACAAATTCAGCCACAATATCACCAAATACAAAATCAGAA
B951c  ...........T.G.C.....................................................C.................C.............
B952c  ...........T.G.C.....................................................C.................C.............
B953c  ...........T.G.C.....................................................C.................C.............
B954c  ...........T.G.C.....................................................C.................C.............
B955cd ...........T.G.C.....................................................C.................C.............
B956a  ...........T.G.C.....................................................C.................C.............
B957ad ...........T.G.C.........................T.............C....T..........C.............
B958c  ...........T.G.C.......................C.............C.................T.............
B959a  ...........T.G.C.....................................C.................T.............
B9510a .........GT.G.C.......................C.....................................T.............
B9511c ...........T.G.C.........................T.............C....T..........C.............
B9512c ...........T.G.C.....................................................C.................C.............
B9513a ...........T.G.C.........................T.............C.................T.............
B9514ad...........T.G.C.........................T.............C....T..........C.............
B9515c ...........T.G.C.....................................................C.................C.............
B9516c ...........T.G.C.....................................................C.................C.............
B971cd ...........T.G.C.......................C.............C.................T.............
B972c  ...........T.G.C.......................C..T...........C.................T.............

RSH86  ACACACCATACAACAGCACAAACCAAAGGCAGAATCACCACTTCAACACAGACCAACAAGCCAAGCACAAAATCACGT
B951c  ..............................G....C...T...C.T.......A...................C.....
B952c  ..................................C...T...C.T.............................C.....
B953c  ..................................C...T...C.T.............................C.....
B954c  ..............................G....C...T...C.T.............................C.....
B955cd ..................................C...T...C.T.............................C.....
B956a  ..................................C...T...C.T........T....................C.....
B957ad ..................................C...T...C..............................C.....
B958c  ..................................C.......C.........A....................C.....
B959a  ..................................C.......C.........A....................C.....
B9510a ..................................C.......C.........A....................C.....
B9511c ..............................G....C...T...C.........A....................C.....
B9512c ..................................C...T...C.T.............................C.....
B9513a ..................................C.......C.........A....................C..T..
B9514ad..................................C...T...C..............................C.....
B9515c ..................................C...T...C.T.............................C.....
B9516c ..................................C...T...C.T.............................C.....
B971cd ........................A.........C.......C.........A....................C.....
B972c  ........................A.....C.......C.........A....................C.....
```

Nucleotide alignment of the partial G gene sequences in this work from all of the RSV B strains 95-98 with Genbank reference strain RSH86OATT (RSH86 bases 4837-5097)

Appendix 5
RSV B Partial G gene amino acid alignment

```
RSH86  NHKVTLTTVTVQTIKNHTGKNISTYLTQVPPERVNSSKQPTTTSPIHTNSATISPNTKSETHHTTAQTKGRITTSTQTNKPSTKSR
B951c  ..........S......E...T......S....SP.................................T..P..N......P.
B952c  ..........S......E...T......S....SP.................................T..P.........P.
B953c  ..........S......E...T......S....SP.................................T..P.........P.
B954c  ..........S......E...T......S....SP.................................T..P.........P.
B955cd ..........S......E...T......S....SP.................................T..P.........P.
B956a  ..........S......E...T......S....SP.................................T..P.........P.
B957ad ..........S......E...T......S....SP........S.....V..................T..P.........P.
B958c  ................E...T......S....SP.......P..........................T..P..N......P.
B959a  ................E...T......S....SP.......P..........................T..P..N......P.
B9510a ................E...T......S...GSP.......P..........................T..P..N......P.
B9511c ..........S......E...T......S....SP........S.....V..................T..P..N......P.
B9512c ..........S......E...T......S....SP.................................T..P.........P.
B9513a ................E...T......S....SP........S.........................T..P..N......PC
B9514ad..........S......E...T......S....SP........S.....V..................T..P.........P.
B9515c ..........S......E...T......S....SP.................................T..P.........P.
B9516c ..........S......E...T......S....SP.................................T..P.........P.
B971cd ................E...T......S....SP.......P.........................S.T..P..N......P.
B972c  ................E...T......S....SP.......PS.......................D.T..P..N......P.
```

Amino acid alignment of the partial G gene sequences in this work from all of the RSV B strains 95-98 with Genbank reference strain RSH86OATT (RSH86 amino acids 1612-1699)

Chapter 6

Concluding remarks

General summary

RSV is a well recognised cause of severe respiratory illness in certain subsets of the population, namely infants, the hospitalised and the elderly (22, 74). Little is known about RSV infection in the general community especially in the adult population. The conclusion of the work reported here was that RSV is a severe cause of illness in all age groups of the community.

It has been demonstrated, in this work, that multiplex PCR can be applied to large numbers of clinical specimens, reducing the time spent on diagnosis or surveillance of several pathogens. Although time consuming, the optimisation of a PCR assay can greatly improve the sensitivity and clarity of the results obtained. The optimisation of a multiplex PCR is inherently more time consuming than that of an individual PCR. For this work the assay was required to be as sensitive as possible and therefore optimisation was essential. Multiplex PCR significantly reduces the consumable costs for surveillance and diagnosis, as effectively one PCR can be performed in the place of many. However, the method of detection of the amplicons will become an important consideration, should the number of targets to be detected increase. Methods other than agarose gel electrophoresis may provide better alternatives. Some of these alternatives were briefly explored in this work, although further testing would be necessary before these could be implemented.

The work reported in this thesis will contribute to the overall understanding of RSV circulation in the general community. It can be concluded that RSV causes significant illness in all age ranges of the community. Also, RSV A and B co-circulate with each other and with influenza, in the general community during the winter months. Furthermore the RSV strains circulating within the general community are representative of those circulating infecting the hospitalised infant population.

Further questions remain unanswered however, for example "where does RSV go in the summer?" Is the variation seen within the G gene of RSV due to a selection pressure on the virus, possibly an immune pressure on the virus in this region, or does it represent the pool of viruses which, although different in this region, are genetically stable? (26, 125). Evidence supporting either of these hypothesis can only be gathered by continual surveillance of RSV over a period of years, in all sections of the community.

It is important to know whether the general community, especially the adult community, is infected with the same viral strains as infants and children. This is because any vaccine which is released for RSV will ultimately be aimed at the infant and children populations (60). For example, had the viral strains circulating in the general community been distinct from those seen in the hospitalised infant populations they may have represented a potential pool from which infants and children could be re-infected, post vaccination

The variation in the G region was discussed earlier (Chapter 5) and any vaccine based on this region would need to take account of this variation. Vaccines are currently being developed, based on the G gene of RSV, and any information on the extent of variation within this region is therefore of use (218). The data gathered in this study may contribute to the overall understanding of the extent of variation in this region of the genome.

For studies, such as the one reported here, where the samples are of poor quality and low copy number, the storage of specimens can be critical. For maximum recovery of an RNA virus the stability of the viral RNA in transport media may be crucial. It would be better, of course, for all of the samples to be analysed upon receipt, preferably with the virus grown to high titre in tissue culture. This is not always possible however. Obviously, the longer the time the virus spends in sub-optimal conditions the lower recovery of that virus. Steps to improve subsequent detection of virus after specimen receipt may include putting the sample into guanidinium based buffers before long term storage. Expansion of the knowledge of RSV circulation and strain distribution within the general community will help with any vaccine design and delivery programs.

Summary of possible further work

Multiplex PCR

Multiplex PCR has been demonstrated to be a very useful technique. It is possible to add more primers into the multiplex PCR described here, however this may compromise the sensitivity of the assay. Other pathogens to be detected include other viruses such as rhinoviruses and adenoviruses. However, the genome of adenoviruses is DNA and detection of both DNA and RNA in the same assay may be difficult. However, if developed this methodology would also allow the detection of bacterial pathogens. This would require the collection medium for the swab to preserve both bacteria and viruses.

Detection methods other than agarose gel electrophoresis may be more practical. The methods investigated here may, if developed further, better facilitate the inclusion of more primer pairs into the multiplex PCR, by eliminating the need for amplicons of distinctive size, which is a constraint on primer design.

A problem with the approach used here was that the protocol for the extraction of nucleic acid was time consuming and laborious. Alternative protocols could be investigated which may not only improve detection but also reduce the amount of time for the procedure. Alternatively, automated methods could be investigated which may provide a more standard method for nucleic acid extraction e.g. Qiagen robot, Tecan or Nuclisens automated extraction machines. These methods would ideally lead to the complete automation of the technique, whereby a sample can be tested for a variety of pathogens and the entire sequence of the causative agent read out.

Epidemiology of RSV

The advantages of using multiplex PCR for the analysis of a large number of samples has been demonstrated. Further application of this method would provide more detailed information about the circulation of RSV within the general community. Information on the circulation of RSV B would be of particular interest, as the levels of RSV B detected in the study reported here were low. Better information on the circulation patterns of different pathogens, and of the types of symptoms which can be associated with the different

pathogens, may allow more accurate clinical diagnosis. For this analysis more specific guidelines need to be issued to the GP on the classification of symptoms. It would be useful if other information could be gathered on the patients presenting with respiratory illness, for example, the patient's temperature.

Follow up of the patients would also be useful as the duration of the symptoms was not available for my work. It would be of interest to see if healthy adults infected with RSV have as severe an illness as those infected with influenza. Several factors would be need to be monitored along with the duration of the symptoms, which may include the measurement of cytokines, temperature, requirement for ventilation etc. It would only be possible, however, to conduct these types of studies on volunteers.

With the release of anti-influenza drugs (Relenza) it has become even more crucial for the correct diagnosis of influenza to be made by the GP. My work has highlighted the difficulty in differentiating between influenza and other forms of respiratory illnesses purely on clinical presentation, especially within the adult population. With the cost of these drugs being substantial, any information about the circulation of other pathogens and markers of symptom presentation will be essential to reduce the mis-diagnosing of influenza.

No attempt to monitor the circulation of RSV beyond the winter season was made here. Although hospital reports of RSV to CDSC are very few during the summer, the collection of samples, from the general community, for analysis using the sensitive method of PCR may reveal any background levels of circulation.

Although the work presented here has revealed that RSV accounts for a significant proportion of illness presenting as ILI, there is still a large proportion of ILI remaining un-diagnosed. Investigation of the samples from which no virus was identified by PCR may reveal other significant pathogens circulating within the community during the winter months. Application of a multiplex PCR to detect targets other than RSV and influenza on these samples would be informative.

Strain analysis

The protocol for the detection of RSV reported here would need to be developed further to provide a sufficiently sensitive assay for the detection of RSV from low copy number, poor quality specimens. A strategy which encompases the entire G gene plus another gene may allow for better comparison of Ks/Kn ratios and also Ts/Tv ratios. This type of strategy would also contribute to the overall understanding of the extent of variation seen with these areas of the genome, which could in turn be related to their function. As more sequence information is gathered on the G gene of RSV, the knowledge of the immune selective pressure on this region will increase. Also, the evolution of the RSV viruses will be able to be followed in a more comprehensive fashion. The transmission studies of RSV within families and in hospitals could also contribute to the overall understanding of the circulation of the virus.

It would be of interest to pursue an individual throughout life and ascertain the number of times an infection with RSV occurs. This kind of study would reveal information on several aspects of re-infection including severity, duration, frequency, subtype and strain type.

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